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Full Length Article

Composition and Morphology of Cuticular Waxes on the Spikes, Flag Leaf Blades and Flag Leaf Sheaths of Wheat (*Triticum aestivum*)

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Abstract

In order to investigate the chemical composition and morphology of cuticular waxes of the three organs (spike, flag leaf and leaf sheath) of wheat (*Triticum aestivum* L.), the glossy variety (Chinese spring) and glaucous variety (Bob white) were selected in this study. Scanning electron microscopy (SEM) revealed the morphology of cuticular wax crystal, such as platelets wax crystals were on the adaxial side of the leaf surfaces, while the spike (glume) and leaf sheath surfaces comprised of tubules wax crystals. Gas Chromatography-Mass Spectrometry (GC-MS) technology analysis displayed five different compound classes on the extracts of the above three organs, including alkane homologues (C25-C33), primary alcohol homologues (C22-C32), aldehyde homologue (C24-C30), fatty acids homologue (C22-C28) and diketones (C31). Interestingly, fatty acids were not presented in the cuticular waxes of the leaf sheath. The total wax contents on the three organs of Bob white were higher than that of Chinese spring. Furthermore, the chemical constituents and relative proportions of the five compound classes significantly differed in different organs and the cultivars. Diketones constituted of β- diketones and OH-β-diketones, and OH-β-diketones were only identified in a glaucous variety (Bob white). At the same time, there were also dramatic changes in the chain length distribution of wax composition. In the waxes of the flag leaves, C28 (octacosanol) was the most prominent. However, C31 β- diketone was the most prominent in the wax compositions of the spikes and flag leaf sheaths. Therefore, based on the above results, this study provided comprehensive information on wheat cuticular waxes and advanced the knowledge of wheat waxes.

Keywords: Cuticular waxes; Morphology; Wheat; GC-MS; β-diketones

Introduction

The terrestrial plants surfaces are covered by the cuticle, and the cuticle is made up of cuticular waxes and a cutin polymer matrix (Jeffree, 2006). Cuticular waxes are of interest to biologist as they have multiple roles in plants, such as insect destruction, protection of plants against ultraviolet (UV) radiation, restricts non-stomatal water loss and pests attack (Eigenbrode and Espelie, 1995; Solovchenko and Merzlyak, 2003; Domínguez *et al.*, 2011).

The epidermis wax is complex mixtures, which is composed of very-long-chain fatty acids (VLCFAs, Chain length>C20), including, alkanes, alcohols, ketones, aldehydes, esters and fatty acids (Jetter *et al.*, 2006). Other components are also found in wax mixtures, such as p-hydroxycinnamic acids, monoacylglycerols (Yonghua*et al.*, 2007), flavonoids (Samuels *et al.*, 2008), methylalkylresorcinols alkylresorcinols (Adamski *et al.*, 2013), benzyl, phenethyl esters (Rapley *et al.*, 2004), and triterpenoids (Javelle *et al.*, 2011; Belge *et al.*, 2014). The cuticular wax compositions of plants are differed from species to species. In some cases, the wax composition tends to be different between the organs of the same species (Racovita *et al.*, 2015). For example, in wheat, the cuticular waxes are comprised of diketones, ester, alkanes, aldehydes and alcohols (Wang *et al.*, 2015a). However, on tomato leaves, the cuticular waxes are mainly composed of n-alkanes, branched alkanes, primary alcohols and triterpenoids (Wang *et al.*, 2015b).

The leaves, stems, and fruits of land plants are coated by cuticles. In the most cases, cuticular waxes are usually present in the form of microcrystals. In the past 60 years, scanning electron microscopy (SEM) has been widely used in observing themorphology of wax crystals (Koch and Ensikat, 2008). Twenty-three wax crystal types have been identified from 13,000 species. In the past few years, several studies have examined the wax crystal of the wheat glume and leaf surfaces. Koch *et al.* observed platelets wax crystals

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on the two-month-old wheat leaves (Koch *et al.*, 2006). Tubule wax crystals have been observed on the wheat glume during grain filling period (Wang *et al.,* 2015a). Although the composition of epidermis wax and wax crystal have been studied in wheat (Bianchi and Figini, 1986; Adamski *et al.,* 2013; Zhang *et al.*, 2013; Wang *et al.*, 2015a; Koch *et al.*, 2006), it remains unclear the differences of cuticular wax composition and crystals among the varieties and organs. The aim of this research was to investigate the differences in the wax patterns in three organs of two wheat varieties Chinese spring (glossy variety) and Bob white (glaucous variety) differing in wax deposition.

Materials and Methods

Plant Material and Reagents

Seeds of Wheat (*Triticum aestivum* L.) varieties (Chinese spring and Bob white) were kindly provided and identified by College of Agronomy, Northwest A&F University, China. Wheat seeds were grown in the research field during the 2017–2018 wheat-growing seasons in Yangling, Shaanxi Province, China (34°19'N, 108°02'E). A total of 90 seeds per variety were individually hand-planted in a 1.5-m row at 10-cm spacing. Standard cultural practices for wheat were followed during the cultivation. Both Chinese spring and Bob white had winter growth habit. The average precipitation, minimum and maximum temperatures from October 1st 2017 to June 1st 2018 in this region were 735.1 mm, – 9.5°C and 24.5°C. The natural precipitation could meet the conditions of wheat growth. Plants were fertilized every two months. Flag leaves, spikes and leaf sheaths were excised from mature plants using clean razor blades during the grain filling stage at April 2018.Exact areas of flag leaf and leaf sheath were determined by photographing them, and then the area was calculated by the ImageJ software. Exact weights of spikes were taken by weighing after extraction (dry weight). N, O-bis (trimethylsilyl)-trifluoroacetamide (BSTFA) (Sigma, USA) and pyridine (Sigma, USA) were used for derivatization reactions. GC-MS (GCMS-QP2010, SHIMADZU, Japan) and GC-FID (GC-2010 PLUS, SHIMADZU, Japan) were used to identify the components of wheat cuticular waxes.

Scanning Electron Microscopy (SEM)

All wheat materials were dried in the desiccators for five days at 55°C. 5 mm^2 completely dried pieces were attached with double adhesive tape to the Aluminium stubs and sputter-coated with gold particles using 90-s bursts from a sputter coater. Coated samples were observed using SEM (Hitachi S4800, Tokyo, Japan), and the working distance was 8.0 mm, the accelerating voltage was 10 kV. First, started with the 400× objective and increased until the coated sample was focused; then, quickly switched to a much higher objective (1200,000×), and slowly turned the coarse focus knob until started to see the image come into focus; Last, each coated sample was detected at 300,000× and 10,000× objective, respectively.

Isolation of Cuticular Waxes and Derivatization Reactions

Cuticular waxes were extracted by using chloroform. Wheat samples were immersed in a glass beaker containing 20 mL chloroform and 20 µg n-tetracosane (C24) as an internal standard. The samples were shaken three times for 1 min at 25°C. After that, each wax sample was filtered through a filter paper, transferred to a GC auto sampler vial and dried under a stream of nitrogen gas. For the next analysis, each wax sample was derivatized with 30 µL pyridine and30µL BSTFA for 1 h at 70°C, and vortexed the wax sample every 20 min. The purpose of this step was to transform hydroxyl (OH-) containing compounds into their corresponding trimethylsilyl derivatives (Schulz *et al.*, 2000). Samples of β-diketones, primary alcohols isolated from wheat leaves were derivatized similarly in the previous study (Adamski *et al.*, 2013). Later on, the wax sample was dried under nitrogen gas, and added 700 µL of chloroform for the GC analysis.

Chemical Analysis of Cuticular Waxes

The GC equipped with an MS detector (MSD) and a flame ionization detector (FID) was used for determining the qualitative and quantitative analysis of wax. Wax composition was analyzed on a capillary GC column (length = 60 m, i.d. = 0.32 mm, df = 0.25 µm) and attached to an MS, programmed to temperature program as follows:set at 50°C for 2 min, ramp 20°C /min to 220°C, held for 2 min, ramp 1.6°C/min to 310°C, held for 18 min at 310°C. All wheat samples were analyzed in triplicates, the coefficient of variance (C.V.) was less than 10% for the three samples.

Statistical Analysis

All the results in our study denote the means ± SD. Each wax component load was calculated based on peak area of each compound and that of the C24. All pictures were drawn with Sigma plot 14.0 software. Statistical analyses to study on cuticular wax composition were performed by using Independent sample T test in SPSS 19.0 software.

Results

Morphology of Wax Crystals on Wheat Plant Parts

The glossy variety (Chinese spring) and glaucous variety (Bob white) of wheat were used in this study. Chinese spring and Bob white showed different cuticular wax phenotypes (Fig. 1). Both the adaxial and abaxial sides of

the flag leaf blade was covered by cuticular waxes. Two types of wax crystals were identified on the leaf surface: platelets and tubules (Fig. 2). Similarly, on the adaxial side of the leaf surface, platelet wax crystals were identified both on Chinese spring (Fig. 2A and 2B) and Bob white (Fig. 2E and 2F). Contrarily, on the abaxial side of the leaf surface, only Bob white was covered with tubule wax crystals (Fig. 2G and 2H). Because the spikes of wheat were covered by glumes, we also observed the glume surfaces by SEM. As shown in Fig. 3, both the glume and leaf sheath surfaces were covered with tubule wax crystals, and Bob white tended to contain denser tubule crystals.

Identification of Cuticular Waxes on Different Organs of Wheat

The GC chromatogram revealed that both Chinese spring and Bob white were constituted of many chemical compounds, including alkanes (a1-a5), primary alcohols (p1-p6), aldehydes (c1-c4), fatty acids (f1-f4) (not present on the leaf sheaths) and diketones (b1 and b2) (Fig. 4 and 5). Their corresponding retention times and carbon chain lengths were identified in the extracts of wheat samples (Table 1). Diketones were composed of β- diketones and OH-β-diketones, with carbon chain length being C31. Among them, OH-β-diketones were only identified in a glaucous variety (Bobwhite). Because fatty acids were not presented in the leaf sheaths of the two wheat varieties, and the flag leaves and spikes contained more compounds than that of the leaf sheaths (Fig. 4 and 5).

Differences in Wax Composition between Cultivars and Organs of Wheat

In the present work, although the spike, flag leaf and leaf sheath cuticular waxes were found to share the most compound classes, they differed significantly in the relative proportions of these compositions.

Wax composition in the spikes: The total wax load on the wheat spike (Bob white) was approximately 1012.58±96.39μg/g, which was much higher than that on Chinese spring (819.91±113.66μg/g) (Table 2 and 3). The wax extracted from spikes (Bobwhite) was dominated by diketones (891.53±92.32 μg/g), representing 88.05% of the total compounds. They were accompanied by low amounts of primary alcohols (4.91%, 49.73±3.87 μg/g) and alkanes (4.38%, 44.32±10.97 μg/g). Relatively small portions of aldehydes (1.97%, 19.95±2.64 μg/g) and fatty acids (0.70%, 7.06±0.58 μg/g) were also presented (Table 3). Differently, in Chinese spring, there were not only lots of diketones (33.46%, 274.34±51.38 μg/g) but also higher amounts of alkanes (38.59%, 316.42±40.52 μg/g), together with substantial amounts of primary alcohols (21.47%, 176.01±23.86 μg/g). Relatively small portions of aldehydes (5.36%, 43.92±5.99 μg/g) and fatty acids (1.12%, 9.21±2.09 μg/g) were also presented (Table 2).

Wax composition in the flag leaves: The total wax load on flag leaves (Bob white) was approximately 73.70±7.70 μg/cm^2, which was five times greater than that of Chinese spring (Table 2 and 3). Diketones (especially OH-β-diketones) and primary alcohols were the two major compound classes that caused the differences between Bob white and Chinese spring. In the cuticular waxes of Bob white leaves, the amounts of diketones and primary

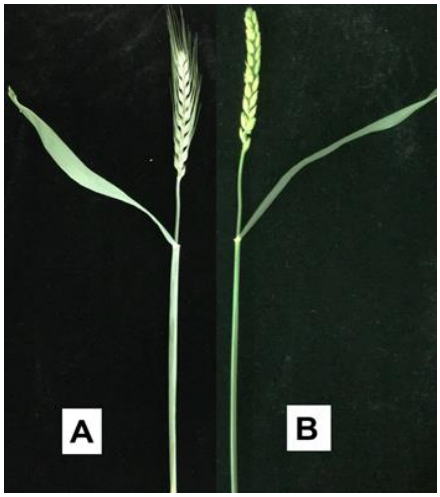

Fig. 1: Cuticular wax phenotype on the flag leaf blade, spike and flag leaf sheath of the two wheat varieties. Glossy variety of Chinese spring (B), and glaucous variety of Bob white (A)

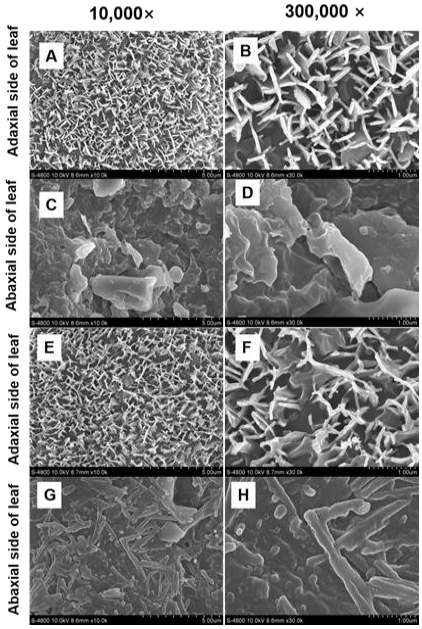

Fig. 2: Epicuticular wax crystals patterns on the adaxial and abaxial side of the leaf surfaces of the two wheat varieties. Glossy variety of Chinese spring (A-D), and glaucous variety of Bob white (E-H). (A, C, E, G) These epicuticular wax crystals were detected at 10,000× magnification, Scale bars = 5 μm; (B, D, F, H). These epicuticular wax crystals were detected at 300,000× magnification, Scale bars = 1 μm

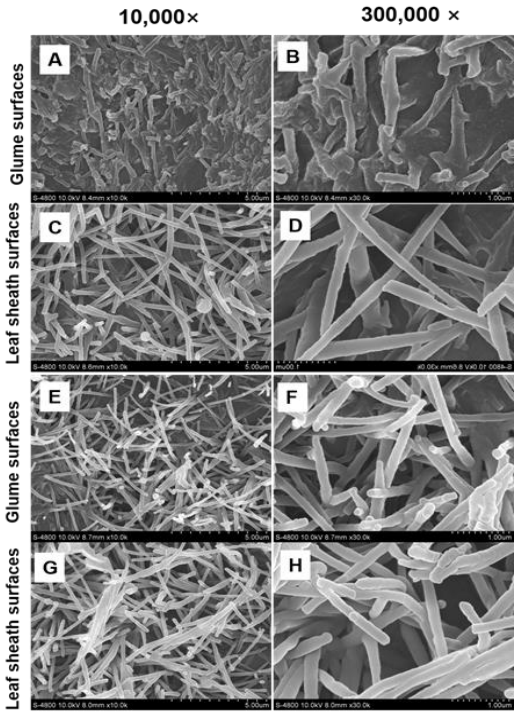

Fig. 3: Epicuticular wax crystals on the glume surfaces and leaf sheath surfaces of the two wheat varieties. Glossy variety of Chinese spring (A-D), and glaucous variety of Bob white (E-H). (A, C, E, G) These epicuticular wax crystals were detected at 10,000×magnification, Scale bars = 5 μm; (B, D, F, H) These epicuticular wax crystals were detected at 300,000×magnification, Scale bars = 1 μm

alcohols were 20.75±6.22 μg/cm^2 (28.15%) and39.37±4.98 μg/cm^2 (53.42%) respectively.

However, Chinese spring exhibited lower amounts of diketones (3.31%, 0.48±0.03μg/cm^2) and primary alcohols (57.17%, 8.22±0.14μg/cm^2) compared with Bob white. The other unusual feature of the waxes of Chinese spring leaves was the higher proportions of alkanes content (35.49%). Inaddition, the rest of the compound classes (aldehydes, fatty acids) were recorded also in lower contents in the two varieties.

Wax composition in the leaf sheaths: Fatty acids were not identified in the leaf sheaths of Bob white and Chinese spring. Diketones were the major compound in the two varieties, especially in the leaf sheaths of Bob white, and diketones represented83.35% of the total compounds. The total wax load of the leaf sheath (Bob white) was approximately 54.70±5.47 μg/cm^2, which was five times greater than that of Chinese spring (Table 2 and 3). Diketones were mainly responsible for the differences between them. In the leaf sheath of Bob white, the content of diketones was up to45.59±4.10 μg/cm^2, while Chinese spring contained lower amounts of diketones (7.27±0.65 μg/cm^2). In addition, the contents of alkanes, aldehydes and primary alcohols were lower in the two varieties.

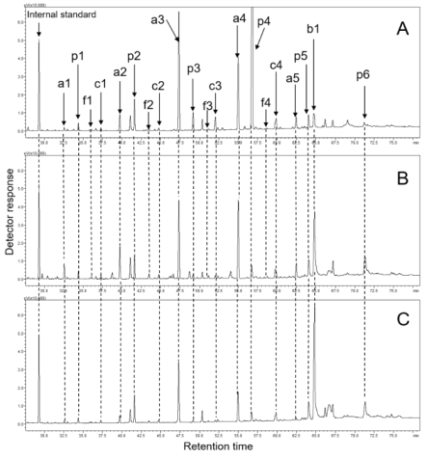

Fig. 4: The GC chromatogram of the Chinese spring.(A) Flag leaf blade; (B) Spike; (C) Flag leaf sheath. a1-a5, Alkanes; p1-p6, Primary alcohols; c1-c4, Aldehydes; f1-f4, Fatty acids; b1-b2, Diketones.The corresponding information of the composition was listed in Table 1

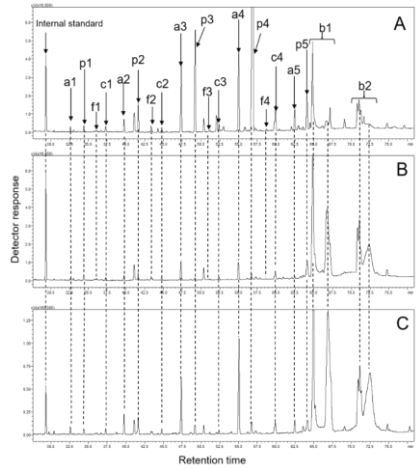

Fig. 5: The GC chromatogram of the Bob white. (A) Flag leaf blade; (B) Spike; (C) Flag leaf sheath. a1-a5, Alkanes; p1-p6, Primary alcohols; c1-c4, Aldehydes; f1-f4, Fatty acids; b1-b2, Diketones. The corresponding information of the composition was listed in Table 1

Chain Length Distributions of the Wheat Compound Classes

The primary alcohols on the spikes, leaves and leaf sheaths of the two wheat cultivars, consisted of even numbers of carbon chain length ranging from C22 to C32 (Fig. 6 and 7). Interestingly, a shift in the dominant primary alcohols chain length was observed in the different organs of the two wheat cultivars. In the flag leaf blades of Chinese spring and Bob white, C28 was the dominant primary alcohol carbon chain length (Fig. 6B and 7B). However, in the spikes and leaf sheaths, C32 (dotriacontanol) was the dominant primary alcohol in

Table 1: Composition and retention time of main waxes identified in the extracts of wheat samples

Compunds	Retention time (min)	Number	Name	Carbon chain length
Internal standard	29.353		tetracosane	24
	32.607	a1	pentacosane	25
	39.764	a2	heptacosane	27
Alkanes	47.374	a3	nonacosane	29
	55.041	a4	hentriacontane	31
	59.935	a5	tritriacontane	33
	34.407	b1	docosanol	22
	41.659	b2	tetracosanol	24
Primary alcohols	49.251	b3	hexacosanol	26
	57.016	b4	octacosanol	28
	64.140	b5	triacontanol	30
	71.225	b6	dotriacontanol	32
	37.327	c1	tetracosanal	24
Aldehydes	44.775	c2	hexacosanal	26
	52.394	c3	octacosanal	28
	59.935	c4	triacontanal	30
	36.107	p1	docosanoic acid	22
	43.374	p2	tetracosanoic acid	24
Fatty acids	51.181	p3	hexacosanoic acid	26
	58.723	p4	octacosanoic acid	28
	64.892	b1	β-diketone	31
	67.201	b1	β-diketone	31
Diketones	70.836	b2	OH-β- diketone	31
	71.272	b2	OH-β- diketone	31
	71.718	b2	OH-β- diketone	31

Chinese spring (Fig. 6A and 6C) while C30 (triacontanol) was prominent in Bob white (Fig.. 7A and 7C). In addition to that, a series of alkanes (C25 to C33) were identified in all organs and varieties, with a relatively broad and odd chain length distribution. The dominant alkane was C29 (nonacosane) in Chinese spring (Fig. 6), but C31 (hentriacontane) became the dominant alkane in Bob white (Fig. 7). Reduced amounts of aldehydes (C24 to C30) were also identified in all the organs and varieties, with C30 being the most prominent (Fig. 6 and 7). Interestingly, fatty acids were not identified on the leaf sheathes. They were only identified on the leaves and spikes, and consisted of even numbers carbon chain length ranging from C24 to C30 (Fig. 6 and 7). The amounts of fatty acids were the lowest in all identified compounds, with C24 (tetracosanoic acid) being the dominant fatty acids in Bob white (Fig. 6 and 7). Diketones were identified as β- diketone and OH-β-diketone, with carbon chain length of C31. OH-β-diketone was only identified in a glaucous variety (Bob white), and the content of β-diketone was always higher than that of OH-β-diketone (Fig. 7).

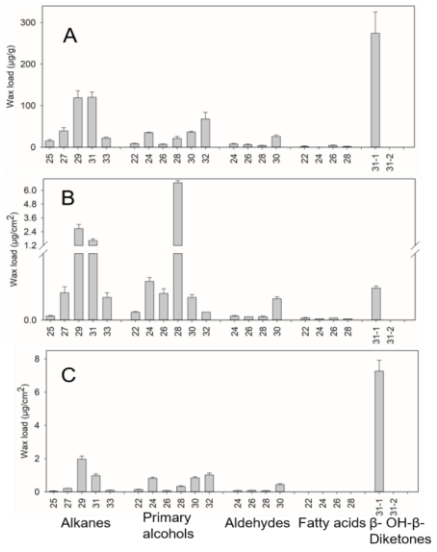

Fig. 6: Cuticular wax compositions on the spikes, flag leaf blades, and flag leaf sheathes of glossy variety Chinese spring. (A)Spikes; (B) Flag leaf blades; (C) Flag leaf sheaths.Each waxconstituent is designated by carbon chain length and is labeled by chemical class along the x-axis. Each value represents the mean of three replicates. Errorbars = SD

Discussion

Although there were some researches on wheat epidermis waxes, many of those studies paid close attention to either whole plants or only leaves of various wheat cultivars without distinguishing the organs of the wheat plant (Tulloch and Weenink, 1969; Bianchi *et al.*, 1980), only the latter research quantified wax loads per surface area (Racovita *et al.*, 2007; Wang *et al.*, 2015a), thus this is the first detailed and comprehensive (compositional and morphological) comparisons of cuticular waxes from the three organs (spike, flag leaf and sheath) of the two wheat cultivars.

The content of total wax on flag leaves of Bob white was five times higher than that of Chinese spring (Table 2 and 3). In Bob white, the spike and leaf sheath waxes were

Table 2: Content and relative contents of cuticular waxon the spike, flag leaf blade and leaf sheath of Chinese spring

Compounds	Content			Relative content (%)		
	Spike (μg/g)	Leaf (μg/cm^2)	Leaf sheath(μg/cm^2)	Spike	Leaf	Leaf sheath
Alkanes	316.42±40.52a	5.10±0.68b	3.30±0.23b	38.59	35.49	22.78
Primary alcohols	176.01±23.86ab	8.22±0.14a	3.24±0.52b	21.47	57.17	22.34
Aldehydes	43.92±5.99c	0.48±0.04c	0.67±0.09c	5.36	3.34	4.66
Fatty acids	9.21±2.09d	0.10±0.01d	np	1.12	0.68	np
Diketones	274.34±51.38a	0.48±0.03c	7.27±0.65a	33.46	3.31	50.21
Total	819.91±113.66	14.38±0.83	14.48±1.53	100.00	100.00	100.00

np, not present. The data are expressed as the mean± SD of three biological replicates. Different letters in columns for each characteristic are significantly different ($P < 0.05$)

Table 3: Content and relative contents of cuticular waxon the spike, flag leaf blade and leaf sheath of Bob white

Compounds	Content			Relative content (%)		
	Spike (μg/g)	Leaf (μg/cm^2)	Leaf sheath (μg/cm^2)	Spike	Leaf	Leaf sheath
Alkanes	44.32±10.97b	10.30±1.73b	5.76±0.75b	4.38	13.98	10.53
Primaryalcohols	49.73±3.87b	39.37±4.98a	2.15±0.15c	4.91	53.42	3.93
Aldehydes	19.95±2.64c	2.65±0.26c	0.97±0.10d	1.97	3.60	1.77
Fatty acids	7.06±0.58d	0.63±0.05d	np	0.70	0.85	np
Diketones	891.53±92.32a	20.75±6.22b	45.59±4.10a	88.05	28.15	83.35
Total	1012.58±96.39	73.70±7.70	54.70±5.47	100.00	100.100	100.00

np, not present. The data are expressed as the mean± SD of three biological replicates. Different letters in columns for each characteristic are significantly different ($P < 0.05$)

dominated by C31 β- and OH-β-diketones (approximately 83%), while leaf waxes contained more than 50% primary alcohols (Table 3). Differently, in Chinese spring, the spike waxes were dominated by alkanes (38%) and C31 β-diketones, flag leaf sheath waxes were dominated by C31 β-diketones (50 %) and alkanes, while leaf waxes contained more than 57% primary alcohols (Table 2).

Epidermis wax layer that covers leaves, stems and spikes, gives the plant surface a glaucous or glossy appearance (Jenks and Ashworth, 1999; Koch and Ensikat, 2008). Koch *et al.* (2008) suggested that platelet crystals are positively correlated with alcohols on the wheat leaf surface. In this paper, dense platelet crystals were found on the adaxial side of the leaf surface of both in Chinese spring and Bob white, and at the same time, high amounts of primary alcohols were also identified in the leaves. Previous researches have demonstrated that tubules structures are dominated by secondary alcohol or β-diketone. The β-diketone tubules mainly contribute to the glaucous phenotype in wheat (Bianchi and Figini, 1986; Adamski *et al.*, 2013; Zhang *et al.*, 2013). Our results showed that Bob white (glaucous variety) and Chinese spring (glossy variety) were covered with wax tubules on glume and leaf sheath (Fig. 3), while the abaxial side of the leaf surface of Chinese spring (glossy variety) displayed a anomalistic wax film (Fig. 2C and 2D). The presence of diketones in all wax samples indicated that β- diketones could be detected both in the glaucous and glossy cultivars (Fig. 6 and 7), but OH-β- diketones could be only identified in the glaucous cultivar (Fig. 7). These results provided an indication of the contents of β-diketone must reach a certain threshold before tubule wax crystals present on the wheat surfaces. For instance, when the β-diketones content reached to 7.27±0.65μg/cm^2, tubule wax crystals presented on the leaf sheath surface (Chinese spring).

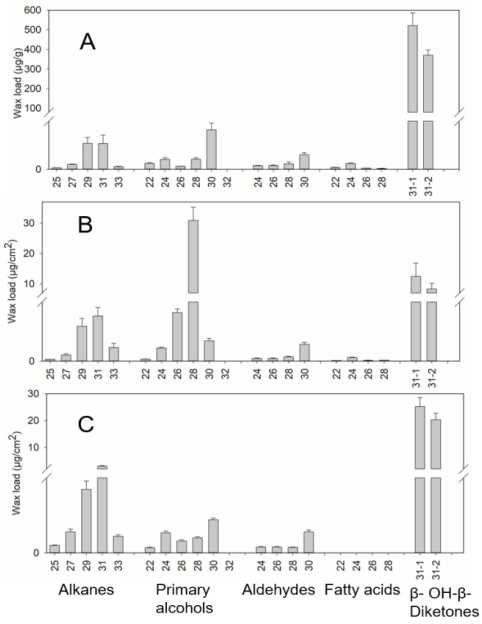

Fig. 7: Cuticular wax compositions on the spikes, flag leaf blades, and Flag leaf sheathes of glossy variety Bob white. (A)Spikes; (B) Flag leaf blade; (C) Flag leaf sheaths. Each wax constituent is designated by carbon chain length and is labeled by chemical class along the x-axis. Each value represents the mean of three replicates. Error bars = SD

Another interesting finding of this study was that the OH-β-diketones were the major compounds contribute to glaucous phenotype in wheat. The glaucous variety was observed in wheat on condition that OH-β-diketones presented in the cuticular waxes. Consequently, based on

the above discussion, it is inferred that β-diketones and OH-β-diketones were synthesized by different enzymes, whilst Chinese spring and Bob white were useful materials for the further studies on the biosynthesis of diketones. Although β-diketones and OH-β-diketones have been found in an increasing number of organisms (Adamski *et al.*, 2013; Zhang *et al.*, 2013; Wang *et al.*, 2015a; Wang *et al.*, 2017), a broader understanding of their bioactivities and their underlying mechanism is lacking. Further studies should be conducted to elucidate the related enzymes and functions in different varieties. More physiological experiments are needed in future studies.

Conclusion

Wax crystals were mainly comprised of platelets on the leaf surface, while they were comprised of tubules on the spike (glume) and leaf sheath. The OH-β-diketones were the major compounds that contributed to the glaucous phenotype in wheat, and the appearance of tubule wax crystals was based on high amounts of β-diketones. The constituents and relative proportions of the wax compound classes differed dramatically among the spikes, flag leaves and flag sheaths of the two wheat cultivars. Most classes of compounds (except for diketones) occurred as homologous series with long chain profiles in different organs and cultivars of wheat.

Acknowledgements

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