Plant Cell Tiss Organ Cult (2017) 128:347–356
DOI 10.1007/s11240-016-1114-2

ORIGINAL ARTICLE

Overexpression of Sorghum *WINL1* gene confers drought tolerance in *Arabidopsis thaliana* through the regulation of cuticular biosynthesis

Shu-Guang Bao[1,2] · Jian-Xin Shi[3] · Feng Luo[1] · Bo Ding[1] · Jin-Yu Hao[1] · Xiao-Dong Xie[1] · Shou-Jun Sun[1]

Received: 1 August 2016 / Accepted: 10 October 2016 / Published online: 20 October 2016

Abstract Plant cuticle plays a significant role in responses to various environmental stresses. Transcription factors were shown to regulate cuticular biosynthesis in many plants. However, no transcription factor genes involved in this biological process have been identified in wax- and cutin-rich sorghum so far. Here we clone and characterize a sorghum gene encoding an ethylene response factor (ERF) transcription factor. It consists of 204 amino acids and holds typically conserved 'mm', 'cm' and AP2 domains of WIN protein family with higher similarity to its orthologues in monocot plants. We designated this gene as sorghum *WIN1-Like 1* gene (*SbWINL1*). Our study showed that *SbWINL1* gene was highly expressed in leaf, stem, seedling and sheath, compared to root and spikelet. Notably, rosette leaves of *SbWINL1*-overexpressed *Arabidopsis* (SbWINL1-OE) were more enriched in wax crystals and shinier than those of wild-type plants. Further biochemical analysis indicated that SbWINL1-OE leaves showed 2- or 2.6-fold higher levels of total wax content or total cutin than counterparts from wild-type leaves. The drought tolerance of SbWINL1-OE plants was enhanced substantially compared with wild-type. Importantly, the expression of genes associated with wax and cutin synthesis pathways was significantly up-regulated in SbWINL1-OE plants. In summary, we demonstrate that the overexpression of sorghum *WINL1* gene in *Arabidopsis* promotes the accumulation of wax and cutin and thus enhances drought tolerance.

Keywords Sorghum · *SbWINL1* · Cutin · Wax · Biosynthetic pathway · *Arabidopsis*

Abbreviations

WIN1	WAX INDUCER1
ERF	Ethylene response factor
SbWINL1	Sorghum *WIN1-Like 1* gene
VLCFAs	Very-long-chain fatty acids
TFs	Transcription factors
FA	Fatty acid
ALK	Alkane
OL	Alcohols
ALD	Aldehydes
2HFA	2-OH fatty acid
DFA	Dioic acid
ω-HFA	ω-OH fatty acid
LACS	Long-chain acyl-CoA synthetase
CER	*ECERIFERUM*

Introduction

Sorghum belongs to the family Poaceae and is a high quality food and forage crop. Sorghum has enriched wax and cutin structures, which play important roles in responses to stress

Electronic supplementary material The online version of this article (doi:10.1007/s11240-016-1114-2) contains supplementary material, which is available to authorized users.

✉ Xiao-Dong Xie
xiex@tjau.edu.cn

✉ Shou-Jun Sun
sunshoujun@tjau.edu.cn

[1] Department of Agronomy, Tianjin Agricultural University, Tianjin 300384, China

[2] Department of Plant Biology and Ecology, College of Life Sciences, Nankai University, Tianjin 300071, China

[3] National Center for Molecular Characterization of Genetically Modified Organisms, School of Life Science and Biotechnology, Shanghai Jiao Tong University, Shanghai 200240, China

and in its adaptability to the external environments, such as drought and water logging (Palmer 1992; Hwang et al. 2002). The molecular mechanism underlying the synthesis and regulation of wax and cutin in sorghum remains largely elusive, although some key genes involved in this process have been identified in model species.

The cuticular layer is composed of cutin and wax and important to the land plants (Riederer 2006; Raffaele et al. 2008; Schreiber 2010). The epidermal wax can be divided into epicuticular wax and intracuticular wax, which are deposited outside of the cuticle and in the cuticular mixtures, respectively (Pollard et al. 2008; Yeats and Rose 2013; Go et al. 2014). The compositions of cuticular cutin and wax vary among species, organs and tissues. Cuticular wax is a mixture of lipids mainly composed of very-long-chain fatty acids (VLCFAs), primary and secondary alcohols, aldehydes, ketone, alkanes and wax esters (Broun et al. 2004; Samuels et al. 2008), whereas cutin is largely composed of hydroxy, epoxy and dicarboxylic fatty acids (Pollard et al. 2008; Go et al. 2014). Cutin and wax are vital elements for the regulation of epidermal permeability and non-stomatal water loss (Schreiber 2010; Burghardt and Riederer 2003), and play crucial roles in protecting plants against insects, pathogens, UV light, and frost (Fiebig et al. 2000). In addition, it was reported that the content of wax is associated with pollen fertility and other agronomic traits (Aarts et al. 1995; Jung et al. 2006).

Genes associated with the cutin and wax pathways have been identified in the past decades (Kunst and Samuels 2009; Borisjuk et al. 2014). Several genes encoding enzymes in wax biosynthesis were also characterized, such as *CER1, CER4, CER6*/*CUT1, KCS1, WAX2, FATB, FAE1* and *FDH* gene (Aarts et al. 1995; Rowland et al. 2006; Millar et al. 1999; Todd et al. 1999; Chen et al. 2003; Pruitt et al. 2000; Mao et al. 2012). For instance, the *CER4* gene encoded a fatty acyl-CoA reductase, which catalyzes the two-step reduction of VLCFAs to primary alcohols (Aarts et al. 1995). *CER6* gene has been shown to be essential for the formation of VLCFAs longer than 22 carbons in *Arabidopsis* stems (Fiebig et al. 2000), while *CER1* and *WAX2*/*CER3* genes are involved in the decarbonylation pathway responsible for the formation of aldehydes, alkanes, secondary alcohols and ketones (Chen et al. 2003; Bernard et al. 2012; Bourdenx et al. 2011; Rowland et al. 2007). Similarly, genes involved in cutin biosynthesis were reported in *Arabidopsis*, such as long-chain acyl-CoA synthetase 1 (*LACS1*), *LACS2*, cytochrome P450 monooxygenase (*CYP86A8*), *CYP86A2*, glycerol-3-phosphate acyltransferase 4 (*GPAT4*), *GPAT8, BODYGUARD* and *HOTHEAD* gene (Lu et al. 2009; Schnurr et al. 2004; Duan and Schuler 2005; Wellesen et al. 2001; Li et al. 2007; Kurdyukov et al. 2006a, b). Among them, *LACS1* is a member of the long chain acyl-CoA synthetase (LACS) family, which is involved in free fatty acids to CoA process (Lu et al. 2009). In addition, *ABCG11 and ABCG12(CER5)* have been proposed to act in cuticular lipid export in *Arabidopsis* (Sieber et al. 2000; Bird et al. 2007; Panikashvili et al. 2007; Bird 2008).

Transcription factors (TFs), including MYB and AP2/ERF TFs, play an important role in the regulation of wax synthesis. *AtMYB41* encodes a R2R3-type MYB involved in regulating wax transport, while AtMYB96 modulates wax accumulation through regulating wax biosynthetic enzymes such as *KCS1, KCS2, KCS6, KCR1, ECERIFERUM1* (*CER1*) and *CER3* (Seo et al. 2011). WAX INDUCER1 (WIN1)/SHINE1, containing an AP2 domain, is the first transcription factor identified as a cuticle biosynthesis regulator in *Arabidopsis* (Broun et al. 2004). Overexpression of *AtWIN1* results in significant accumulation of wax by up-regulating the expression of many genes in the wax biosynthetic pathway. Furthermore, *AtWIN1* has been shown to trigger wax production, enhance drought tolerance and modulate cuticular permeability when it was overexpressed in *Arabidopsis* (Aharoni et al. 2004). Cutin was significantly increased in vegetative and reproductive organs in *Arabidopsis* plants overexpressing *AtWIN1*, while the opposite effects were observed when *AtWIN1* expression was down-regulated. Therefore, a two-step process was proposed for the *AtWIN1* regulation of cutin and wax production (Kannangara et al. 2007). An in-depth study suggested that *SHN1* (*AtWIN1*) and its other two *SHINE* (*SHN*) clade members act redundantly to shape the surface and morphology of *Arabidopsis* flowers via controlling cuticular lipid metabolism and modifying the epidermis cell wall (Shi et al. 2011a, b). Recently, it has been demonstrated that *WIN1* regulates epidermal cell morphology and cuticle development coordinately with MYB106 and MYB16 transcription factors in *Arabidopsis* and *Torenia fournieri* (Oshima et al. 2013). More recently, it has been shown that the *WIN1* gene is closely related to drought and defense responses in plants (Sela et al. 2013; Wang et al. 2012; Al-Abdallat et al. 2014).

However, no transcription factor responsible for cuticular biosynthesis in sorghum has been identified so far and the mechanism underlying this process remains to be determined. Here, we isolate a transcription factor gene *SbWINL1* from sorghum and investigate its function in *Arabidopsis*. Our study demonstrated that *SbWINL1* gene is highly expressed in leaf, stem, seedling and sheath, particularly in leaf and stem of sorghum. Overexpressing *SbWINL1* in *Arabidopsis* substantially enhanced the drought tolerance via the accumulation of wax and cutin. Consistently, genes related to cutin and wax biosynthesis pathway were up-regulated in the transgenic plants. Our study will further facilitate the understanding of the biological role of *WIN1* orthologous genes, and shed light on the biosynthetic and regulatory pathways of cutin and wax in sorghum.

Materials and methods

Plant materials

Arabidopsis plants (Col-0 ecotype) were grown under 22 °C, 75% relative atmospheric humidity, and 16/8 h photoperiod (day/night) with light intensity of 200 µmol m^{-2} s^{-1}. Sorghum plants (line BTx622) were cultivated in a growth room with 25 °C, 50% relative humidity, and 16/8 h photoperiod (day/night) with light intensity of 200 µmol m^{-2} s^{-1}.

Construction of expression vector and screening of transgenic plants

The coding region of *SbWINL1* gene was PCR-amplified from leaf cDNA of sorghum by using specific primer pair as follows: ATGGTACAGCCAAAGAAG and TCAGATGATGAAGCGACC. To study the biological functions of *SbWINL1*, an overexpression vector was constructed through the Gateway cloning technology. Firstly, the target gene containing the AttB sites was obtained by PCR amplification with AttB sites added at the 5′ end of the primers. The PCR product was recombined into the entry vector pDONR221 (Amp) through BP reaction. Secondly, the target gene was replaced into overexpression vector pGWB14 (Kan) by LR reaction, and under the control of 35S promoter. The resulting plasmid was introduced into *Agrobacterium* strain GV3101, and transformed into *Arabidopsis* plants by the floral dip method. Positive transgenic plants were then selected on 1/2 MS medium containing 50 mg L^{-1} = kanamycin.

Environment scanning electron microscopy (ESEM) analysis

For cuticular wax structure analysis, fresh leaves from four-week-old wild type and transgenic *Arabidopsis* plants were placed in a clean petri dish and dried at room temperature for 2–3 days. Leaf samples were then treated with gold sputtering and examined by a Quanta250FEG (ESEM mode) scanning electron microscope (FEI).

Analysis of wax and cutin composition

For wax and cutin analysis, each sample with about ten mature rosette leaves of four-week-old *Arabidopsis* plants was used for extraction of wax and cutin. The analysis protocol of wax and cutin contents was described in detail in previous studies (Kurdyukov et al. 2006a; Shi et al. 2011; Franke et al. 2005). The main instruments for above analysis are GC-MS (Agilent Technologies) and GC-FID (Agilent Technologies).

Drought treatment

Seven-day-old seedlings of wild type and SbWINL1-OE, grown on 1/2 MS medium, were transplanted into nutrient soil for another 7 days, then drought treatment was carried out for 20 days (Zou et al. 2015; Liu et al. 2016; Wang et al. 2016). The survival rates of plant were measured 3 days after rewatering.

Gene expression analysis

For expression pattern analysis of *SbWINL1* gene, quantitative real time PCR (q RT-PCR) was performed as follows. Total RNA was extracted from root, 10-day-old seedling, stem, leaves, sheath and spikelet of sorghum, respectively. 2 µg of total RNA was used for first-strand cDNA synthesis by SuperScript III transcriptase (Invitrogen). Sorghum *β-ACTIN* gene acted as the internal control. For the expression analysis of wax and cutin related synthetic genes and *SbWINL1* gene in SbWINL1-OE plants, 2 µg of total RNA was extracted from 4-week-old rosette leaves of SbWINL1-OE and wild type *Arabidopsis* plants. The first-strand cDNA was then synthesized using SuperScript III transcriptase (Invitrogen). *ACTIN2* gene was quantified as an internal control.

The resultant cDNA solution was used for semi-quantitative PCR and qRT-PCR. The qRT-PCR analysis was performed using an Applied Biosystems 7500 real-time PCR system. The SYBR *Premix Ex Taq* Kit (TakaRa) was used for reaction according to the manufacturer's instruction.

Accession numbers

Sequence data from this article can be found in the GenBank/EMBL databases under the following accession numbers: X79378 for Sorghum *β-ACTIN*, At1g01600 for *CYP86A4*, At1g63710 for *CYP86A7*, At1g12570 for *HTH-like*, At1g49430 for *LACS2*, At1g01610 for *GPAT4*, At1g02205 for *CER1*, At4g24510 for *CER2*, At5g57800 for *WAX2*, At1g01120 for *KCS1* and At2g04570.

Results

Cloning and structural analysis of *SbWINL1* from sorghum

The coding region of *SbWINL1* was isolated from the sorghum stem. It contains 615 bp and encodes 204 amino acids. Sequence analysis showed a highly conserved AP2 domain and two WIN protein characteristic 'mm' and 'cm' domains (Aharoni et al. 2004; Shi et al. 2013), suggesting SbWINL1 from sorghum is a typical WIN protein, (Fig. 1a). Further

sequence comparison indicated an 85 % sequence identity to OsWR1 of rice, 81 % to BdWIN1 of *Brachypodium distachyon* and 75 % to HvNud of *Hordeum vulgare*, though a much lower sequence identity to VvWIN1of *Vitis vinifera* (64 %). Overall, SbWINL1 presented higher homology with WIN1 proteins from the monocots than dicots. The phylogenetic tree analysis showed that SbWINL1 shared high similarity to orthologous proteins from rice and *Brachypodium distachyon* (Fig. 1b).

Expression profile analysis of *SbWINL1* gene and functional evaluation in *Arabidopsis*

To investigate the spatial expression pattern of *SbWINL1*, we performed quantitative real-time PCR (qRT-PCR) analysis in different tissues from sorghum. Our data showed that *SbWINL1* was expressed in root, seedling, leaf, stem, sheath and spikelet (Fig. 2a), with higher expression in leaf, stem, seedling and sheath than root and spikelet. To elucidate the physiological role of the *SbWINL1* gene, we generated transgenic *Arabidopsis* plants (SbWINL1-OE) with the *SbWINL1* gene controlled by the CAMV 35S promoter (Fig. 2b). After validation of the transgenic plants, we chose T2 transgenic *Arabidopsis* plants for subsequent studies. The expression levels of SbWINL1-OE-5# and SbWINL1-OE-9# were analyzed using semi-quantitative RT-PCR methods and the results showed that *SbWINL1* gene overexpressed in transgenic *Arabidopsis* plants while there were no transcripts of foreign gene detected in wild type plants (Fig. 2c). Strikingly, SbWINL1-OE plants exhibited much glossier rosette leaves compared with control plants (Fig. 3), suggesting that the overexpression of *SbWINL1* might influence the synthesis pathway of cuticular wax. Similar phenotypes were found in AtWIN1-OE *Arabidopsis* plants (Broun

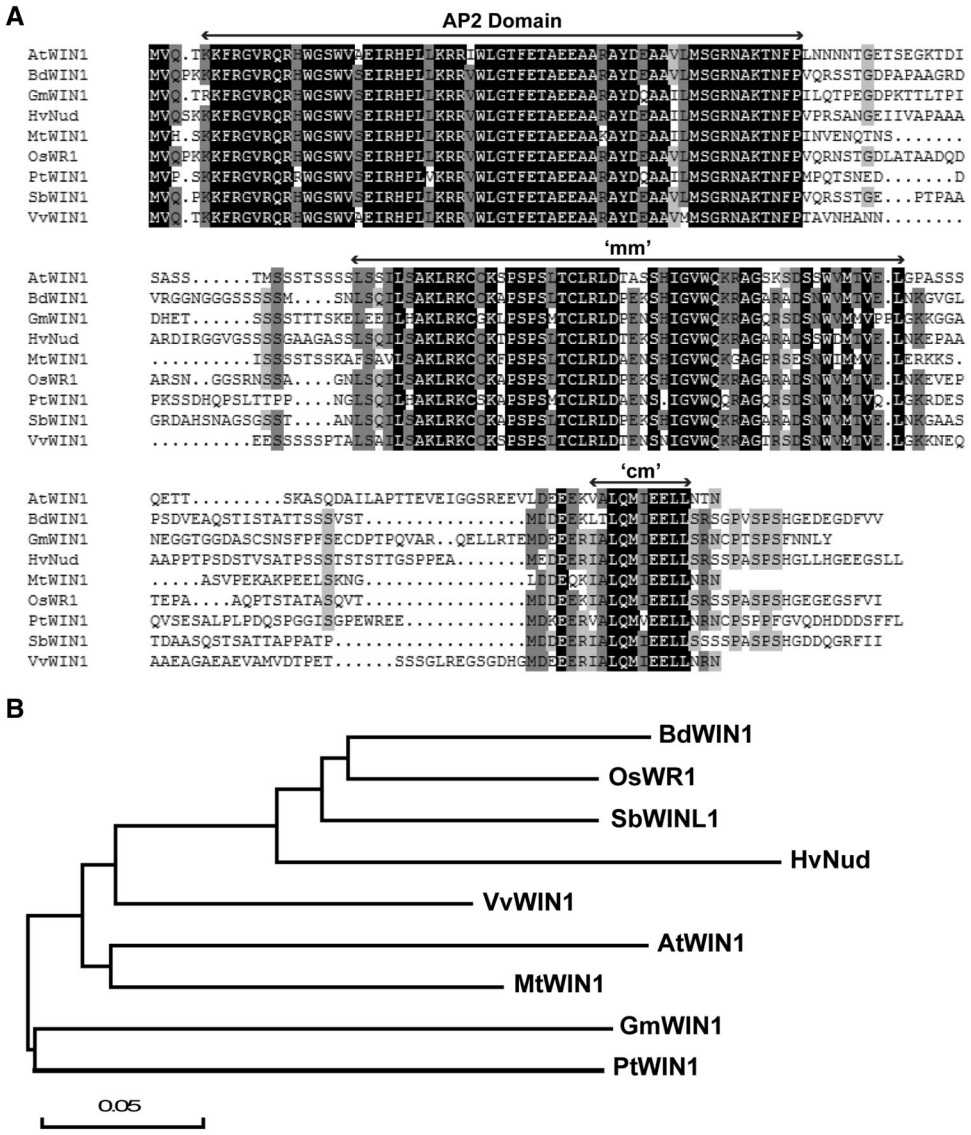

Fig. 1 Comparative analysis of WIN1 proteins in various plant species. **a** Multiple alignment analysis of WIN1 protein sequences. All nine proteins contain a single AP2 domain at their N termini, a conserved middl1e domain (termed mm), and a conserved C-terminal domain (termed cm). *White letters* shaded indicate amino acids that are either 100 % identical (*black*) or identical in at least 75 % (*dark gray*) or identical in at least 50 % (*light gray*) of all proteins. Vv *Vitis vinifera*; Mt *Medicago truncatula*; At *Arabidopsis thaliana*; Bd *Brachypodium distachyon*; Os *Oryza sativa Japonica Group*; Sb *Sorghum bicolor*; Gm *Glycine max*; Pt *Populus trichocarpa*; Hv *Hordeum vulgare*. **b** Phylogenetic tree analysis of WIN1 proteins

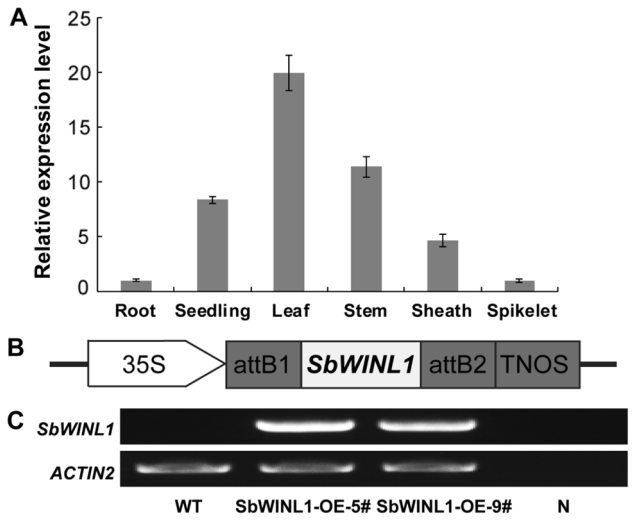

Fig. 2 Expression level of *SbWINL1* in *Sorghum* and transgenic *Arabidopsis*. **a** Detection of expression levels of *SbWINL1* gene in various tissues of sorghum using qRT-PCR. **b** Schematic representation of the *SbWINL1* over-expression construction. 35S, 35S promoter; TNOS, NOS terminator. **c** Detection of expression levels of *SbWINL1* gene in WT, SbWINL1-OE-5# and SbWINL1-OE-9#. Semi-quantitative RT-PCR was also performed with *AtACTIN2* primers as control. *SbWINL1* semi-quantitative RT-PCR products are shown after 35 PCR cycles, and actin products are shown after 25 PCR cycles. *N* negative control

et al. 2004; Kannangara et al. 2007), implying a functional similarity between *SbWINL1* and *AtWIN1*.

Overexpression of *SbWINL1* significantly contributes to the accumulation of wax and cutin in *Arabidopsis*

To understand the structural alteration accounting for the observed phenotypes, we examined the leaf surface of SbWINL1-OE plants with ESEM. The results showed that there were more wax crystals and films distributed on the surface of transgenic plant leaves than on wild-type leaves (Fig. 4), supporting the idea that overexpressing *SbWINL1* enhances the synthesis of wax. The chemical analysis of wax and cutin in 4-week-old rosette leaves by GC-FID and GC-MS revealed a significant difference in the amount of wax and cutin between SbWINL1-OE and wild type plants. The total content of wax in leaves from SbWINL1-OE plants was significantly increased up to twofold higher than control plants (Fig. 5a). The main components of wax such as fatty acids, alcohols, alkanes and aldehydes were increased significantly in SbWINL1-OE plants, whereas 16:0 fatty acid, 18:0 fatty acid and 32:0 alcohol were decreased, compared with wild-type controls (Fig. 5c).

It was reported that overexpressing *AtWIN1* can dramatically affect cutin biosynthesis in *Arabidopsis* leaves, so we next assessed whether *SbWINL1* could exert the same effect on cutin biosynthesis. We analyzed the levels of cutin in SbWINL1-OE rosette leaves. The results showed that the total cutin content in transgenic plant leaves was about 2.6-fold higher than control plants (Fig. 5b). Notably, most of the cutin monomers were dramatically increased in SbWINL1-OE plants compared with wild-type controls (Fig. 5d). Also the overexpression of *SbWINL1* significantly promoted the accumulation of C24:0 2-OH fatty acids, C16:0 and C18:2 dioic acid. The contents of ω-hydroxy fatty acids, such as C16:0, C18:0, C18:1 and C18:2, were also substantially increased (Fig. 5d). These results indicated that overexpression of *SbWINL1* contributed to the accumulation of wax and cutin.

Overexpression of *SbWINL1* enhances drought tolerance in *Arabidopsis*

It was reported that *WIN1* gene is involved in drought and defense responses (Sela et al. 2013; Wang et al. 2012; Al-Abdallat et al. 2014). OsWR1 and SlSHN1 transgenic

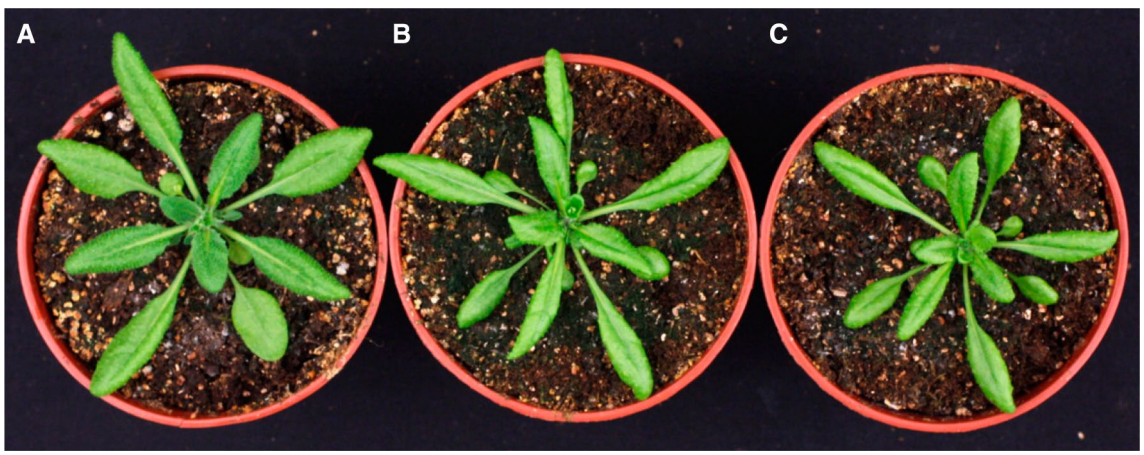

Fig. 3 Glossy leaves of SbWINL1-OE plant. **a***Arabidopsis* wild type (Col-0), **b** SbWINL1-OE-5# and **c** SbWINL1-OE-9#

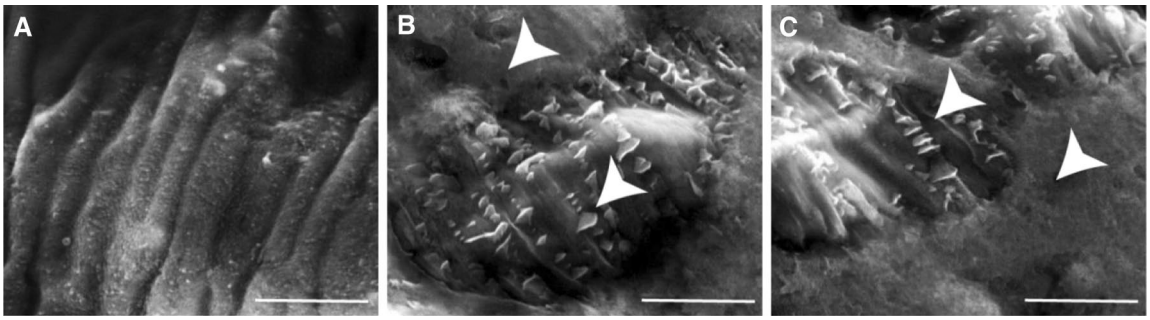

Fig. 4 Environmental Scanning Electron Microscopy analysis of epicuticular wax deposition on the surfaces of rosette leaves in WT (**a**), SbWINL1-OE-5# (**b**) and SbWINL1-OE-9# (**c**). Images were taken at 20,000 magnification (*bar* = 2 μm). The *white arrows* indicate the wax crystals and film

plants were insensitive to drought stress. To examine the sensitivity of SbWINL1-OE plants to drought stress, we performed drought treatment on seedlings of transgenic and wild-type plants. The results showed that SbWINL1-OE *Arabidopsis* was insensitive to drought stress compared with wild- type plants (Fig. 6a). Importantly, the survival rates of SbWINL1-OE *Arabidopsis* plants were significantly higher than those of control plants (Fig. 6b), indicating that overexpression of *SbWINL1* enhances drought tolerance in *Arabidopsis*.

Overexpression of *SbWINL1* up-regulates the expression of wax and cutin related genes in SbWINL1-OE plants

As reported previously, the transcription factor *AtWIN1* triggered the expression of genes involved in the biosynthesis of wax in 35S:WIN1 *Arabidopsis* (Broun et al. 2004; Kannangara et al. 2007). To examine the effect of *SbWINL1* on wax biosynthesis-related genes, we measured the expression of *CER1, CER2, WAX2* and *KCS1*, which are important genes involved in wax biosynthesis, in rosette leaves from 4-week-old *Arabidopsis* by quantitative RT-PCR. The results demonstrated that the expression of four genes related to wax biosynthesis was significantly increased, particularly for *CER1* gene expression (Fig. 7a).

To further validate the notion that the overexpression of *SbWINL1* gene might modulate the expression of genes associated with cutin synthesis in SbWINL1-OE plants, we examined the expression of six cutin synthesis relevant genes with quantitative RT-PCR. As expected, the tested genes were significantly up-regulated in *SbWINL1* overexpressing plants (Fig. 7b). These results indicated that *SbWINL1* plays an important role in regulating the expression of genes involved in wax and cutin biosynthesis and thus enhancing the accumulation of wax and cutin, resulting in glossy and shiny leaves.

Discussion

Wax and cutin play important roles in plant growth, development and in response to environmental stresses (Kunst and Samuels 2009, 2003; Borisjuk et al. 2014; Sieber et al. 2000; Woloshuk and Kolattukudy 1986; Fauth et al. 1996; Preuss et al. 1993; Scott et al. 2004). WIN1 transcription factor has been shown to be a positive regulator of wax and cutin synthesis in some plant species (Broun et al. 2004; Kannangara et al. 2007; Shi et al. 2011; Wang et al. 2012; Al-Abdallat et al. 2014), however there is scarce report on the molecular mechanism of wax and cutin synthesis in sorghum, a crop with enriched wax and cutin in the cuticular layer (Hwang et al. 2002; Lochte-Watson and Weller 1999; Burow et al. 2008; Peters et al. 2009; Lee et al. 2014). In this study, we cloned and characterized the *SbWINL1*gene from sorghum, which encodes an AP2 type transcription factor with high similarity to WIN1 proteins from other plant species (Shi et al. 2011, 2013; Wang et al. 2012; Taketa et al. 2008). It was reported that AtWIN1 transcription factor activates wax and cutin synthesis in *Arabidopsis* and thus contributes to shiny plants (Broun et al. 2004; Kannangara et al. 2007). Here we demonstrated that the overexpression of SbWINL1 in *Arabidopsis* also increased the wax and cutin synthesis and resulted in glossy and shiny plants, consistent with previous reports on its orthologues OsWR1 and AtWIN1 (Broun et al. 2004; Aharoni et al. 2004; Kannangara et al. 2007; Wang et al. 2012).

Chemical analysis revealed that waxes such as C26:0, C30:0, C32:0 and C34:0 fatty acids, campesterol, B-sitosterol and C31:0 alkanes were dramatically increased in SbWINL1-OE plants. Interestingly, although cutin monomers such as C16:0 and C18:2 dioic acids, and all of the ω-hydroxy fatty acids were increased dramatically in SbWINL1-OE plants, unlike *AtWIN1*, there was no significant change in C16:0 and C18:0 fatty acids in cutin monomers, indicating that functional differences might exist between *SbWINl* and *AtWIN1* and this merits further investigation.

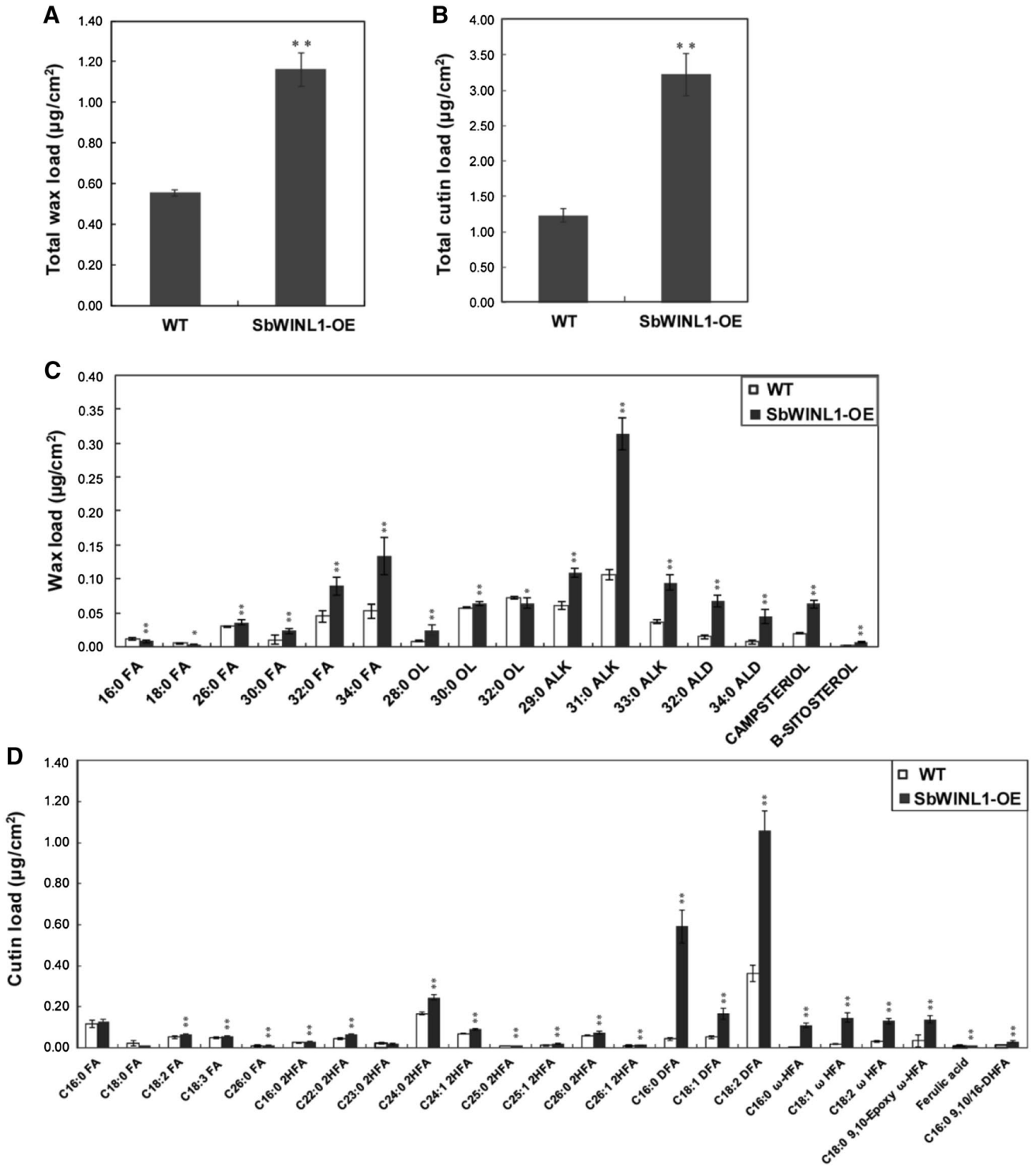

Fig. 5 Changes of wax and cutin in WT and SbWINL1-OE rosette leaves. Rosette leaves of 4-week-old wild-type (Col-0) and SbWINL1-OE plants grown in soil were used for analysis of wax and cutin composition. Each value represents the mean of five replicates. Statistically analyzed using a Student's *t* test (*$0.05 < p < 0.01$, **$p < 0.01$). *Bars* indicate SD of the mean. *FA* fatty acid, *ALK* alkane, *OL* alcohols, *ALD* aldehydes, *2HFA* 2-OH fatty acid, *DFA* dioic acid, *ω-HFA* ω-OH fatty acid, C16:0 9/10 16-DHFA, 9/10 16 di-OH C16 FA. **a** Total leaf wax of SbWINL1-OE and wild type plants. **b** Total leaf cutin of SbWINL1-OE and wild type plants. **c** Wax profiles of SbWINL1-OE and wild type plants. **d** Cutin profiles of SbWINL1-OE and wild type plants

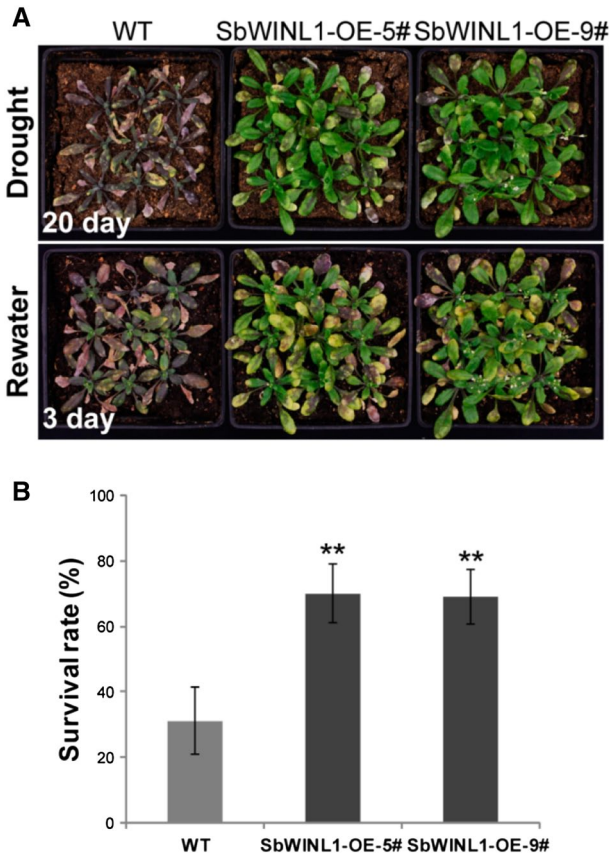

Fig. 6 Drought stress tolerance was enhanced in SbWINL1-OE plants. **a** Reponses of SbWINL1-OE and wild type plants to drought stress. **b** Survival rates of SbWINL1-OE and wild type plants under drought stress. The experiments were repeated three times with similar results. Data shown are mean values ± SD ($n = 36$). Date are statistically analyzed using a Student's t test (**$p < 0.01$)

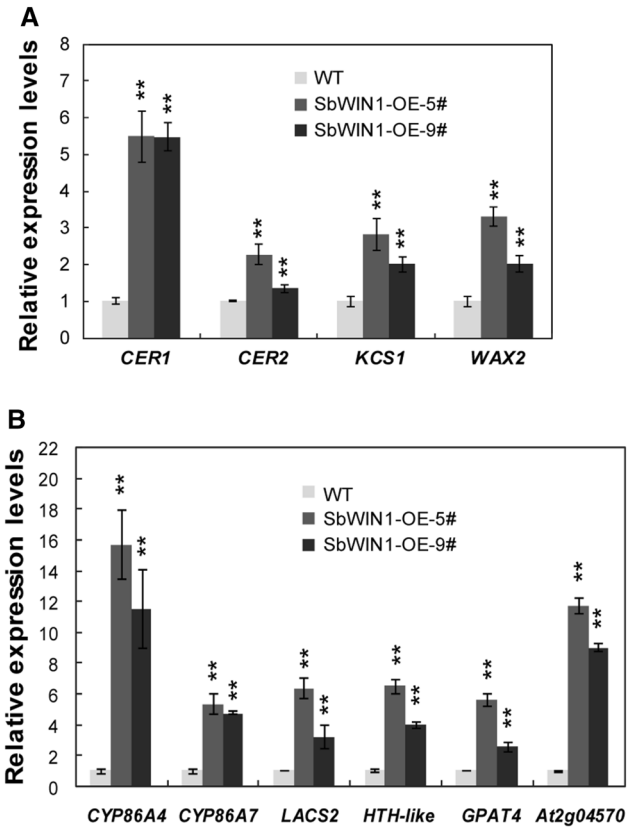

Fig. 7 Changes in the expression levels of genes related to wax (**a**) and cutin (**b**) biosynthesis in SbWINL1-OE plants. Quantitative RT-PCR was carried out to analyze the patterns of gene expression. The experiments were repeated two times with similar results. Data shown are mean values ± SD ($n = 3$). Difference levels among transgenic and wild type plants were statistically analyzed using a Student's t test (**$p < 0.01$)

It was reported that *WIN1* overexpression enhanced drought tolerance and defense responses by increasing wax and cutin accumulation in plants (Sela et al. 2013; Wang et al. 2012; Al-Abdallat et al. 2014). Similarly, overexpression of *SbWINL1* in *Arabidopsis* enhanced drought tolerance compared with wild-type. Statistical analysis showed that the survival rate of SbWINL1-OE was obviously higher than control plants in drought treatment experiments. These results demonstrated that *SbWINL1* enhanced drought tolerance by accumulation of wax and cutin.

Transcription factors can promote or block the transcription of target genes and thus control various biological processes. Several transcription factors have been identified to regulate wax and cutin synthesis by directly or indirectly interacting with relevant target genes (Go et al. 2014; Seo et al. 2011; Kannangara et al. 2007; Oshima et al. 2013). Our study showed that genes known to be involved in the biosynthesis pathway of cutin and wax were up-regulated. Among them, the expression of four wax biosynthetic genes and six cutin biosynthetic genes were dramatically increased in SbWINL1-OE plants. This indicated that overexpression of *SbWINL1* had the same effect on wax and cutin biosynthesis as its *Arabidopsis* orthologues *AtWIN1* (Broun et al. 2004).

Taken together, our study showed that *SbWINL1* functions in a similar manner to its orthologous genes in other species (Broun et al. 2004; Oshima et al. 2013; Sela et al. 2013; Wang et al. 2012; Al-Abdallat et al. 2014) and enhanced the drought tolerance.

Acknowledgments We are grateful to Professor Dabing Zhang and Dr. Dierdre McLachlan for the critical reading of the manuscript and stimulating advice and to Ms. Qian Luo and Dr. Guorun Qu from Shanghai Jiao Tong University for their assistance in the GC-FID and GC-MS analysis. This work was supported by Thousand Talents Program (Physiological and Genetic Mechanism of Sorghum Stress Resistance), Innovative Group Scheme of Tianjin (TD12-5017), Science and Technology Support Program (16YFZCNC00630) and Special Funds of Agricultural Ministry for Public Welfare Professions (201503134).

Author contributions The experiments were designed and supervised by Prof. Shoujun Sun and Prof. Xiaodong Xie. Mr. Shuguang Bao carried out the main experiments, statistical analysis, figure preparation and manuscript editing; Associate Prof. Jianxin Shi performed data acquisition and statistical analysis of wax and cutin measuring

experiments. Mr. Feng Luo and Mrs. Bo Ding performed the gene expression analysis. Mr. Feng Luo, Mrs. Bo Ding and Mrs. Jinyu Hao performed the drought stress tolerance experiment. Prof. Xie and Associate Prof. Shi revised the manuscript. All authors read and approved the manuscript.

Compliance with ethical standards

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

References

Aarts MG, Keijzer CJ, Stiekema WJ, Pereira A (1995) Molecular characterization of the CER1 gene of *Arabidopsis* involved in epicuticular wax biosynthesis and pollen fertility. Plant Cell 7:2115–2127

Aharoni A, Dixit S, Jetter R, Thoenes E, Arkel G, Pereira A (2004) The SHINE clade of AP2 domain transcription factors activates wax biosynthesis, alters cuticle properties, and confers drought tolerance when overexpressed in *Arabidopsis*. Plant Cell 16:2463–2480

Al-Abdallat AM, Al-Debei HS, Ayad JY, Hasan S (2014) Overexpression of SlSHN1 gene improves drought tolerance by increasing cuticular wax accumulation in tomato. Int J Mol Sci 15:19499–19515

Bernard A, Domergue F, Pascal S, Jetter R, Renne C, Faure JD, Haslam RP, Napier JA, Lessire R, Joubès J (2012) Reconstitution of plant alkane biosynthesis in yeast demonstrates that *Arabidopsis* ECERIFERUM1 and ECERIFERUM3 are core components of a very-long-chain alkane synthesis complex. Plant Cell 24:3106–3118

Bird DA (2008) The role of ABC transporters in cuticular lipid secretion. Plant Sci 174:563–569

Bird D, Beisson F, Brigham A, Shin J, Greer S, Jetter R, Kunst L, Wu X, Yephremov A, Samuels L (2007) Characterization of *Arabidopsis* ABCG11/WBC11, an ATP binding cassette (ABC) transporter that is required for cuticular lipid secretion. Plant J 52:485–498

Borisjuk N, Hrmova M, Lopato S (2014) Transcriptional regulation of cuticle biosynthesis. Biotechnol Adv 32:526–540

Bourdenx B, Bernard A, Domergue F, Pascal S, Léger A, Roby D, Pervent M, Vile D, Haslam RP, Napier JA, Lessire R, Joubès J (2011) Overexpression of *Arabidopsis* ECERIFERUM1 promotes wax very-long-chain alkane biosynthesis and influences plant response to biotic and abiotic stresses. Plant Physiol 156:29–45

Broun P, Poindexter P, Osborne E, Jiang CZ, Riechmann JL (2004) WIN1, a transcriptional activator of epidermal wax accumulation in *Arabidopsis*. Proc Natl Acad Sci USA 101:4706–4711

Burghardt M, Riederer M (2003) Ecophysiological relevance of cuticular transpiration of deciduous and evergreen plants in relation to stomatal closure and leaf water potential. J Exp Bot 54:1941–1949

Burow GB, Franks CD, Xin Z (2008) Genetic and physiological analysis of an irradiated bloomless mutant (epicuticular wax mutant) of sorghum. Crop Sci 48:41–48

Chen XB, Goodwin SM, Boroff VL, Liu XL, Jenks MA (2003) Cloning and characterization of the *WAX2* gene of *Arabidopsis* involved in cuticle membrane and wax production. Plant Cell 15:1170–1185

Duan H, Schuler MA (2005) Differential expression and evolution of the *Arabidopsis* CYP86A subfamily. Plant Physiol 137:1067–1081

Fauth M, Merten A, Hahn MG, Jeblick W, Kauss H (1996) Competence for elicitation of H_2O_2 in hypocotyls of cucumber is induced by breaching the cuticle and is enhanced by salicylic acid. Plant Physiol 110:347–354

Fiebig A, Mayfield JA, Miley NL, Chau S, Fischer RL, Preuss D (2000) Alterations in CER6, a gene identical to CUT1, differentially affect long-chain lipid content on the surface of pollen and stems. Plant Cell 12:2001–2008

Franke R, Briesen I, Wojciechowski T, Faust A, Yephremov A, Nawrath C, Schreiber L (2005) Apoplastic polyesters in *Arabidopsis* surface tissues-a typical suberin and a particular cutin. Phytochemistry 66:2643–2658

Go YS, Kim H, Kim HJ, Suh MC (2014) *Arabidopsis* cuticular wax biosynthesis is negatively regulated by the DEWAX gene encoding an AP2/ERF-Type transcription factor. Plant Cell 26:1666–1680

Hwang KT, Cuppett SL, Weller CL, Hanna MA (2002) Properties, composition and analysis of grain sorghum wax. J Am Oil Chem Soc 79:521–527

Jung KH, Han MJ, Lee DY, Lee YS, Schreiber L, Franke R, Faust A, Yephremov A, Saedler H, Kim YW, Hwang I, An G (2006) Wax-deficient anther1is involved in cuticle and wax production in rice anther walls and is required for pollen development. Plant Cell 18:3015–3032

Kannangara R, Branigan C, Liu Y, Penfield T, Rao V, Mouille G, Höfte H, Pauly M, Riechmann JL, Broun P (2007) The transcription factor WIN1/SHN1 regulates cutin biosynthesis in *Arabidopsis thaliana*. Plant Cell 19:1278–1294

Kunst L, Samuels AL (2003) Biosynthesis and secretion of plant cuticular wax. Prog Lipid Res 42:51–80

Kunst L, Samuels L (2009) Plant cuticles shine: advances in wax biosynthesis and export. Curr Opin Plant Biol 12:721–727

Kurdyukov S, Faust A, Nawrath C, Bär S, Voisin D, Efremova N, Franke R, Schreiber L, Saedler H, Métraux JP, Yephremov A (2006a) The epidermis-specific extracellular BODYGUARD controls cuticle development and morphogenesis in *Arabidopsis*. Plant Cell 18:321–339

Kurdyukov S, Faust A, Trenkamp S, Bar S, Franke R, Efremova N, Tietjen K, Schreiber L, Saedler H, Yephremov A (2006b) Genetic and biochemical evidence for involvement of HOTHEAD in the biosynthesis of long-chain alpha-omega- dicarboxylic fatty acids and formation of extracellular matrix. Planta 224:315–329

Lee BH, Carr TP, Weller CL, Cuppett S, Dweikat IM, Schlegel V (2014) Grain sorghum whole kernel oil lowers plasma and liver cholesterol in male hamsters with minimal wax involvement. J Funct Foods 7:709–718

Li Y, Beisson F, Koo AJ, Molina I, Pollard M, Ohlrogge J (2007) Identification of acyltransferases required for cutin biosynthesis and production of cutin with suberin-like monomers. Proc Natl Acad Sci USA 104:18339–18344

Liu S, Wang C, Jia F, An Y, Liu C, Xia X, Yin W (2016) Secretory peptide PdEPF2 enhances drought tolerance by modulating stomatal density and regulates ABA response in transgenic *Arabidopsis thaliana*. Plant Cell Tiss Org 125:419–431

Lochte-Watson KR, Weller CL (1999) Wax yield of grain sorghum (Sorghum bicolor) as affected by mechanical harvesting, threshing, and handling methods. Appl Eng Agric 15:69–72

Lu SY, Song T, Kosma DK, Parsons EP, Rowland O, Jenks MA (2009) *Arabidopsis* CER8 encodes LONG-CHAIN ACYL-COA SYNTHETASE 1 (LACS1) that has overlapping functions with LACS2 in plant wax and cutin synthesis. Plant J 59:553–564

Mao B, Cheng Z, Lei C, Xu F, Gao S, Ren Y, Wang J, Zhang X, Wang J, Wu F, Guo X, Liu X, Wu C, Wang H, Wan J (2012) Wax crystal-sparse leaf 2, a rice homologue of WAX2/GL1, is involved in synthesis of leaf cuticular wax. Planta 235:39–52

Millar AA, Clemens S, Zachgo S, Giblin EM, Taylor DC, Kunst L (1999) CUT1, an *Arabidopsis* gene required for cuticular wax biosynthesis and pollen fertility, encodes a very-long-chain fatty acid condensing enzyme. Plant Cell 11:825–838

Oshima Y, Shikata M, Koyama T, Ohtsubo N, Mitsuda N, Ohme-Takagi M (2013) MIXTA-Like transcription factors and WAX

INDUCER1/SHINE1 coordinately regulate cuticle development in Arabidopsis and *Torenia fournieri*. Plant Cell 25:1609–1624

Palmer GH (1992) Sorghum-food, beverage and brewing potentials. Process Biochem 27:145–153

Panikashvili D, Savaldi-Goldstein S, Mandel T, Yifhar T, Franke RB, Hofer R, Schreiber L, Chory J, Aharoni A (2007) The *Arabidopsis* DESPERADO/AtWBC11 transporter is required for cutin and wax secretion. Plant Physiol 145:1345–1360

Peters PJ, Jenks MA, Rich PJ, Axtell JD, Ejeta G (2009) Mutagenesis, selection, and allelic analysis of epicuticular wax mutants in Sorghum. Crop Sci 49:1250–1258

Pollard M, Beisson F, Li YH, Ohlrogge JB (2008) Building lipid barriers: biosynthesis of cutin and suberin. Trends Plant Sci 13:236–246

Preuss D, Lemieux B, Yen G, Davis RW (1993) A conditional sterile mutation eliminates surface components from *Arabidopsis* pollen and disrupts cell signaling during fertilization. Genes Dev 7:974–985

Pruitt RE, Vielle-Calzada JP, Ploense SE, Grossniklaus U, Lolle SJ (2000) FIDDLEHEAD, a gene required to suppress epidermal cell interactions in *Arabidopsis*, encodes a putative lipid biosynthetic enzyme. Proc Natl Acad Sci USA 97:1311–1316

Raffaele S, Vailleau F, Leger A, Joubes J, Miersch O, Huard C, Blée E, Mongrand S, Domergue F, Roby D (2008) A MYB transcription factor regulates very-long-chain fatty acid biosynthesis for activation of the hypersensitive cell death response in *Arabidopsis*. Plant Cell 20:752–767

Riederer M (2006) Thermo dynamics of the water permeability of plant cuticles: characterization of the polar pathway. J Exp Bot 57:2937–2942

Rowland O, Zheng HQ, Hepworth SR, Lam P, Jetter R, Kunst L (2006) CER4 encodes an alcohol-forming fatty acyl-coenzyme a reductase involved in cuticular wax production in *Arabidopsis*. Plant Physiol 142:866–877

Rowland O, Lee R, Franke R, Schreiber L, Kunst L (2007) The CER3 wax biosynthetic gene from *Arabidopsis thaliana* is allelic to WAX2/YRE/FLP1. FEBS Lett 581:3538–3544

Samuels L, Kunst L, Jetter R (2008) Sealing plant surfaces: cuticular wax formation by epidermal cells. Annu Rev Plant Biol 59:683–707

Schnurr J, Shockey J, Browse J (2004) The acyl-CoA synthetase encoded by LACS2 is essential for normal cuticle development in *Arabidopsis*. Plant Cell 16:629–642

Schreiber L (2010) Transport barriers made of cutin, suberin and associated wax. Trends Plant Sci 15:546–553

Scott RJ, Spielman M, Dickinson HG (2004) Stamen structure and function. Plant Cell 16:S46–S60

Sela D, Buxdorf K, Shi JX, Feldmesser E, Schreiber L, Aharoni A, Levy M (2013) Overexpression of AtSHN1/WIN1 provokes unique defense responses. PLoS One 8:e70146

Seo PJ, Lee SB, Suh MC, Park MJ, Go YS, Park CM (2011) The MYB96 transcription factor regulates cuticular wax biosynthesis under drought conditions in *Arabidopsis*. Plant Cell 23:1138–1152

Shi J, Tan H, Yu XH, Liu Y, Liang W, Ranathunge K, Franke RB, Schreiber L, Wang Y, Kai G, Shanklin J, Ma H, Zhang D (2011a) Defective pollen wall is required for anther and microspore development in rice and encodes a fatty acyl carrier protein reductase. Plant Cell 23:2225–2246

Shi JX, Malitsky S, de Oliveira S, Branigan C, Franke RB, Schreiber L, Aharoni A (2011b) SHINE transcription factors act redundantly to pattern the archetypal surface of *Arabidopsis* flower organs. PLoS Genet 7:e1001388

Shi JX, Adato A, Alkan N, He Y, Lashbrooke J, Matas AJ, Meir S, Malitsky S, Isaacson T, Prusky D, Leshkowitz D, Schreiber L, Granell AR, Widemann E, Grausem B, Pinot F, Rose JK, Rogachev I, Rothan C, Aharoni A (2013) The tomato SlSHINE3 transcription factor regulates fruit cuticle formation and epidermal patterning. New Phytol 197:468–480

Sieber P, Schorderet M, Ryser U, Buchala A, Kolattukudy P, Metraux J, Nawrath C (2000) Transgenic *Arabidopsis* plants expressing a fungal cutinase show alterations in the structure and properties of the cuticle and postgenital organ fusions. Plant Cell 12:721–738

Taketa S, Amano S, Tsujino Y, Sato T, Saisho D, Kakeda K, Nomura M, Suzuki T, Matsumoto T, Sato K, Kanamori H, Kawasaki S, Takeda K (2008) Barley grain with adhering hulls is controlled by an ERF family transcription factor gene regulating a lipid biosynthesis pathway. Proc Natl Acad Sci USA 105:4062–4067

Todd J, Post-Beittenmiller D, Jaworski JG (1999) KCS1 encodes a fatty acid elongase 3-ketoacyl-CoA synthase affecting wax biosynthesis in *Arabidopsis thaliana*. Plant J 17:119–130

Wang Y, Wan L, Zhang L, Zhang Z, Zhang H, Quan R, Zhou S, Huang R (2012) An ethylene response factor OsWR1 responsive to drought stress transcriptionally activates wax synthesis related genes and increases wax production in rice. Plant Mol Biol 78:275–288

Wang F, Zhu H, Chen D, Li Z, Peng R, Yao Q (2016) A grape bHLH transcription factor gene, *VvbHLH1*, increases the accumulation of flavonoids and enhances salt and drought tolerance in transgenic *Arabidopsis thaliana*. Plant Cell Tiss Org 125:387–398

Wellesen K, Durst F, Pinot F, Benveniste I, Nettesheim K, Wisman E, Steiner-Lange S, Saedler H, Yephremov A (2001) Functional analysis of the LACERATA gene of *Arabidopsis* provides evidence for different robes of fatty acid omegahydroxylation in development. Proc Natl Acad Sci USA 98:9694–9699

Woloshuk CP, Kolattukudy PE (1986) Mechanism by which contact with plant cuticle triggers cutinase gene expression in the spores of Fusariumsolanif. Proc Natl Acad Sci USA 83:1704–1708

Yeats TH, Rose JKC (2013) The formation and function of plant cuticles. Plant Physiol 163:5–20

Zou JJ, Li XD, Ratnasekera D, Wang C, Liu WX, Song LF, Zhang WZ, Wu WH (2015) *Arabidopsis* CALCIUM-DEPENDENT PROTEIN KINASE 8 and CATALASE3 function in abscisic acid-mediated signaling and H_2O_2 homeostasis in stomatal guard cells under drought stress. Plant Cell 27:1445–14460