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CRISPR/Cas9-Mediated characterization of Kcs6 in wax biosynthesis and drought tolerance of *Arabidopsis thaliana*

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ABSTRACT

Drought is a major abiotic stress that reduces crop productivity due to global climate change. The plant cuticular wax layer plays a crucial role in reducing non-stomatal water loss and enhancing drought tolerance. KCS6, encoding 3-ketoacyl-CoA synthase 6, is a key enzyme in the biosynthesis of very-long-chain fatty acids (VLCFAs), the main components of cuticular wax. However, its specific function in wax accumulation and drought resistance remains incompletely elucidated. This study aimed to characterize the role of KCS6 in wax biosynthesis and drought tolerance in Arabidopsis thaliana using CRISPR/Cas9-mediated gene knock-out. kcs6 mutants were generated by targeting the first exon of KCS6 to create a loss-of-function allele. Phenotypic analysis, cuticular wax composition, and drought tolerance assays were performed in the mutants compared to wild-type plants. The results showed that kcs6 mutants exhibited a significant reduction in VLCFA content and cuticular wax load. The mutants also displayed a higher rate of water loss and increased sensitivity to drought stress compared to the wild type. These findings provide direct genetic evidence that KCS6 is essential for cuticular wax biosynthesis and for maintaining plant water status under drought conditions. This study is the first to demonstrate, using CRISPR/Cas9-mediated targeted mutagenesis, that disruption of KCS6 directly links altered VLCFA-derived cuticular wax biosynthesis to impaired plant water retention and drought adaptation, providing mechanistic evidence for KCS6 as a promising genetic target for improving crop drought resilience.

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INTRODUCTION

Climate change has led to an increasing frequency and intensity of drought events worldwide, posing a severe threat to agricultural productivity and global food security (IPCC, 2023). Water deficit impairs plant growth, development, and yield by disrupting physiological processes such as photosynthesis, nutrient uptake, and cellular homeostasis (Zandalinas et al., 2021). Many physiological and biochemical pathways are affected by drought stress (Marques & Hu, 2024). Water accounts for 80–95 percent of the plant body's fresh biomass (Ievinsh, 2023) and plays an important role in a variety of physiological activities such as plant growth (Hoenicka et al., 2024), development, and metabolism (Seleiman et al., 2021). Therefore, understanding the molecular mechanisms underlying plant drought tolerance is critical for developing crop varieties adapted to

water-limited environments.

Arabidopsis thaliana is a small rosette plant that grows as a biennial (Adams et al, 2016) or cold-weather annual, part of the Brassicaceae taxonomic family (Meinke et al., 1998). Because Arabidopsis thaliana is a typical glycophyte (Abobata, 2020), and many other xerophytes or desiccation-tolerant plants contain drought-resistant systems analogous to glycophytes (Kosová, Prášil, & Vítámvás, 2019), it is the most often used model organism for studying plant drought tolerance (Van et al., 2009). Combined with a newly designed method for producing gene knockout lines (Decaestecker et al., 2019), has led many fundamental biologists to believe that Arabidopsis is the best model system for basic studies in multicellular eukaryotic biology (Meinke et al, 1998). Water deficiency and saline stress resistance in Arabidopsis plants improved after administration of the non-protein aminobutyric acid (Hussein et al., 2024; Hoenicka et al., 2024), which is reported to generate plant resistance to various diseases (Zabar et al., 2025).

One of the key adaptive strategies in plants to cope with water loss is the formation of a cuticular wax layer on the epidermal surface of aerial organs. The presence of waxes on the surface of plants serves to protect them from desiccation, UV light (Lewandowska et al., 2020), and frost damage, and acts as a barrier to combat diseases and regulate plant-insect interactions (Huang et al., 2022). Wax is made up of very long-chain fatty acids, known as VLCFAs, and their derivatives (Batsale et al., 2021). Wax production begins in the plastid (He et al, 2020), where long-chain fatty acids (LCFAs) are synthesized by a fatty acid synthase (FAS) (Batsale et al., 2021). The major constituents of cuticular wax are very-long-chain fatty acids (VLCFAs) and their derivatives, including alkanes, alcohols, and esters. A fatty acid elongase (FAE) complex transports these synthesized products to the endoplasmic reticulum (ER) (Batsale et al., 2023), where it elongates them into very-long-chain fatty acids (VLCFAs) (Batsale et al, 2021). Plant cuticular waxes are complex combinations of very long-chain fatty acids (VLCFAs) (Kunst & Samuels., 2006) and their derivatives, including aldehydes, alkanes, primary and secondary alcohols, esters, and ketones, that can be fragmented using organic solvents. A fatty acid elongation complex (FAE), which includes 3-ketoacyl-CoA synthases (KCSs), catalyzes these reactions (Kim et al., 2022).

Among the KCS family members in Arabidopsis thaliana, KCS6, also known as CUT1 or ECERIFERUM6, has been implicated in the elongation of C24–C28 fatty acids, which are precursors for major wax components. Transcriptomic and genetic studies have shown that KCS6 is a direct target of drought-responsive transcription factors such as MYB96, and its expression is strongly induced under ABA and water deficit conditions (Seo & Park, 2021; Huang et al., 2022). However, previous studies relying on T-DNA insertion lines often retain partial gene activity or background effects. Consequently, the precise contribution of KCS6 to wax biosynthesis and its direct role in drought tolerance remain to be fully elucidated using precise genome editing.

The advent of CRISPR/Cas9 genome editing technology provides a powerful tool to generate precise loss-of-function mutants with minimal off-target effects. Recent work has demonstrated the application of CRISPR/Cas9 to generate kcs5 and kcs6 mutants, revealing that concurrent mutations of both genes nearly block drought-induced wax production and implicating their key roles in wax biosynthesis during drought (Abualrub et al., 2022). The most commonly used CRISPR/Cas9 and other CRISPR/Cas systems usually cut double-stranded DNA and generate a Double-Strand Break

(DSB) (Zhang et al., 2021). Currently, Agrobacterium-mediated CRISPR/Cas9 genome editing is the most commonly used delivery method (Sandhya et al., 2020); for most plant species, this method can achieve high efficiency compared with other delivery methods (Alok et al., 2020). By creating a clean knock-out of KCS6, it is possible to directly assess its function in wax accumulation and physiological responses to drought stress without the limitations of traditional mutagenesis approaches.

In this study, we employed CRISPR/Cas9 to generate kcs6 knock-out mutants in Arabidopsis thaliana. We characterized the mutants for cuticular wax composition, water loss rate, and drought tolerance phenotypes. Our findings provide direct genetic evidence that KCS6 is essential for VLCFA-derived wax biosynthesis and plays a crucial role in maintaining water homeostasis under drought conditions (Abualrub et al., 2022; Huang et al., 2022). This work not only advances our understanding of cuticle biology but also identifies KCS6 as a potential target for molecular breeding of drought-resilient crops (Hamdan et al., 2022; Zhang et al., 2023).

METHOD

Plant Materials and Stress Conditions

Seeds of Arabidopsis thaliana were used in this study, acquired from the Cotton Biotechnology Laboratory, University of Agriculture Faisalabad, and cultivated in a growth room under controlled conditions. Seeds were grown on Murashige and Skoog MS plates and placed in a growing chamber set to 22°C (Drake et al., 2016). All well-germinated seedlings were transplanted to new pots 2 weeks after planting. Photoperiods (8 hours of darkness and 16 hours of light) and the optimal temperature (22°C) were observed. 3 weeks after transplanting, all of the plants had reached their vegetative maturity stage, as evidenced by four fully developed true leaves and no visible flower buds.

Construction of KCS6 vector and plasmid isolation

To investigate the activity of KCS6, Bacterial strain GV3101 was used in our study. The KCS6 (Huang et al., 2022) vector was constructed and consists of Plant Codon-Optimized Cas9, which is triggered by the 35S promoter of the Cauliflower Mosaic Virus and guarantees effective expression in plants. The HindIII sites on either side of the 1.2 kb Spectinomycin Resistance Gene (McCue et al, 2025) facilitate cell selection and possible removal. Essential components such as an origin of replication, selectable markers (such as antibiotic resistance genes), a Multiple Cloning Site (MCS), and BsaI recognition sites for accurate cloning are included in the 16.5 kb Vector Backbone. Liquid cultures for plasmid isolation were created by selecting a single, overnight-developed bacterial colony and inoculating 3mL of LB broth with the appropriate selection of antibiotics. Culture tubes were incubated in a shaker (180 rpm) overnight at 37°C (Zhang et al, 2006). Alkaline lysis solutions were used to extract the plasmid DNA (Li, Li, & Sheen, 2010). The purity of the plasmid DNA was determined using an agarose gel. Plasmid DNA was extracted from small-scale bacterial colonies.

Preparation of Chemically Competent Cells of Agrobacterium Reagents

10 mL of LB medium containing Rifampicin and gentamicin was added to a single colony of the GV3101 strain and incubated at 28°C overnight in a shaker. 50 mL of LB broth was used to inoculate a 250 mL flask holding a 30 L primary culture (containing 50 mL of gentamicin and

rifampicin). Overnight at 28°C, the flask was shaken until a thick, hazy growth appeared and the OD reached 0.04. It was chilled for 8 minutes on ice after reaching the desired OD.

Transformation of vector into competent Agrobacterium cells

Before receiving 1 titer of plasmid DNA, competent cells were placed on ice and frozen for five minutes. The vial was submerged in liquid nitrogen for five minutes. After 5 minutes, the competent cell vial was kept in a water bath at 37 °C. 250 L competent cells and 1 mL of LB medium were shaken for two hours in a 15 mL Corning tube. The mixture was centrifuged for 2 minutes at 5000 rpm. Medium with 6–200 titers was used to resuscitate the cells. Seven LB agar plates containing cells received the addition of Rifampicin and Gentamicin. Plates were kept at 28 °C for two days to encourage colony growth.

Floral dipping transformation

The plant seeds were grown for around 2.5 weeks on MS plates before being transplanted to soil-filled pots. To maintain a high moisture level, the pots were covered with plastic bags. Both the tray and the pots must be kept sufficiently moist. After a couple of days, the tray's cover and water were removed, but not the pots. Cut the primary inflorescence as soon as it appears to encourage the development of the secondary inflorescence, and attempt to do it before the bloom opens. Secondary inflorescence development was observed when it developed before floral emergence altered it. This was performed by vigorously shaking 5 mL of the agrobacterium cultured in media containing related antibiotics, such as hygromycin, rifampicin, gentamicin, and kanamycin, overnight at 28 °C. The O.D. was 1.2 to 1.5. Following overnight shaking, it was centrifuged and then dissolved in 2 ml of agrofiltration medium. Once more, the O.D. was between 0.8 and 1.2. The flowers were dipped in it, giving them a tip. Following the transformation, wrap the pots in plastic and cover them. The same bed was transformed once more after two weeks.

Quantification of Cuticular wax

Wax was extracted from the aerial parts of 5-week-old Arabidopsis plants. Inflorescence stems and cauline leaves were used because they accumulate the highest wax load (Fiebig et al., 2000). The plant's transformation, q/RTPCR was used. Chloroform will be placed on Petri plates; mutant and regular, completely inflated leaves will be dipped in the plates for 30 seconds to measure the wax. There will be 3-5 leaves per sample; the average of these leaves will be used to determine the correct wax content. The percentage reduction in wax of the KCS6 knockout Arabidopsis plant ranges from 41% to 51% by comparing 10 wild-type and 10 mutant plants.

RESULTS AND DISCUSSION

Ligation of sgRNA into KCS6 vector

The annealed oligos of gDNA with BsaI restriction site-based sticky ends were ligated with the gel-eluted backbone of the GV301 vector. The GV301-sgRNA-Cas9 construct was ready to be transformed into a full plant expression vector.

Table 1 presents the restriction enzyme analysis of the KCS6 recombinant vector using the double-cutter restriction enzyme HindIII to verify the integrity of the plasmid construct. Two HindIII recognition sites were identified at 1,576 bp and 16,082 bp, generating an expected restriction

fragment of 14,506 bp between the two cleavage sites. Based on the total plasmid size of 16,087 bp, digestion with HindIII was predicted to produce two DNA fragments of approximately 14,506 bp and 1,581 bp. The observed fragment sizes are consistent with the expected restriction map, indicating that the recombinant plasmid carrying the KCS6 insert was correctly assembled.

Table 1. KCS6 vector restriction confirmation with restriction enzyme HindIII (Double cutter)

Site 1	**16082bp**
Site 2	1576bp
Difference in band	16082bp-1576bp = 14506bp
Total cut from original size of vector	14506bp
Overall size of vector	16087bp
Remaining band size of vector	16087-14506 = 1581bp

Restriction enzyme digestion remains one of the most reliable methods for validating recombinant plasmids before downstream applications such as plant transformation, sequencing, or gene expression analyses. By comparing the observed restriction pattern with the theoretical restriction map generated during vector design, researchers can rapidly determine whether the target insert has been successfully incorporated and whether major plasmid rearrangements have occurred. Recent molecular cloning protocols continue to recommend restriction digestion as an essential quality-control step because it provides rapid, cost-effective confirmation of plasmid identity prior to more detailed sequence verification (Green & Sambrook, 2021; Addgene, 2023).

The presence of the expected 14.5-kb fragment indicates that the HindIII recognition sites remained intact following insertion of the KCS6 gene. Likewise, the predicted 1.58-kb fragment corresponds to the remaining portion of the plasmid outside the larger restriction fragment, further supporting the structural integrity of the recombinant construct. Agreement between the predicted and observed restriction fragments suggests that no major deletions, insertions, or recombination events occurred during plasmid construction. Similar approaches are routinely employed in recombinant DNA technology to confirm successful vector assembly before functional characterization of cloned genes (Green & Sambrook, 2021; SnapGene, 2024).

Transformation of constructs into Agrobacterium cells

The constructions were chosen on LB medium containing 100 bp, 400 bp, and 500 bp. 1, 2, 3, 4, and 5 for ligation confirmation with 52 rifampicin, kanamycin, and gentamicin, as mentioned in Figure 1. After that, the constructions were streaked on LB plates for miniprep confirmation.

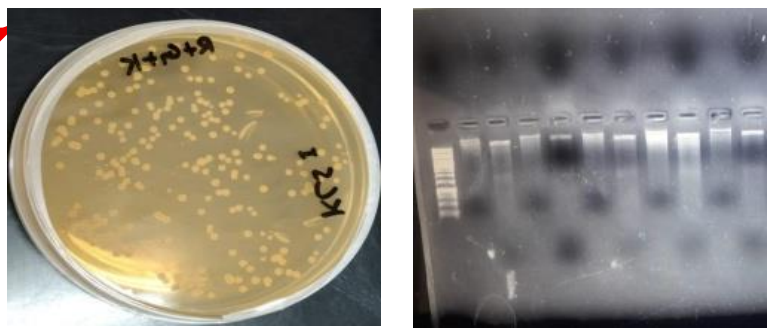

Figure 1. Agrobacterium GV3101 was used to transform the confirmed constructs, which were then selected on LB plates with antibiotics and confirmed by colony PCR and miniprep

Confirmation of KCS6 Plasmid in Agrobacterium

Plant genetic engineering uses Agrobacterium's ability to transfer DNA to plant cells. The plasmid from the GV301-transformed Agrobacterium cells was extracted and run on a 1% agarose gel. The presence of plasmid KCS6 with a band size of 16.6 kilobytes was confirmed.

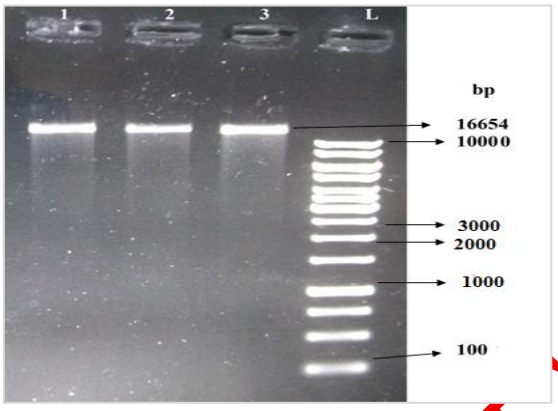

Figure 2. KCS6 plasmid confirmation on 1% agarose gel. Wells 1, 2, and 3 contain plasmids from distinct colonies of transformed agrobacterium cells, and L is the 1kb ladder

Confirmation of Restriction Digestion of KCS6

The KCS6 plant expression vector with plant codon-optimized Cas9 and a 35S promoter was obtained from Addgene. The plasmid contains a 1.2kb spectinomycin gene with HindIII restriction sites. KCS6 was digested using BsaI (a type IIS restriction enzyme that can be used to create DNA fragments with distinctive overhangs for directed cloning). It cuts DNA at specified recognition sites but cleaves at a predetermined distance away from these sites.

Certain DNA fragments are produced during the digestion of the KCS6 vector with BsaI, which can be isolated and examined) enzyme and then run on a 1% agarose gel. On the Gel documentation system, bands were confirmed. The existence of two bands of 1.2kb and 16.5kb proved the presence of a limited vector backbone.

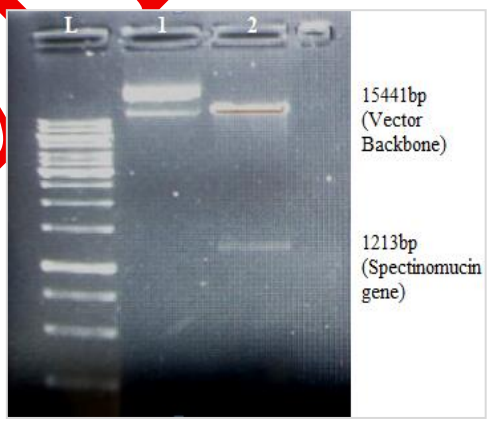

Figure 3. Confirmation of KCS6 restriction with HindIII

This enzyme flanked the streptomycin resistance gene in the KCS6 plasmid. The vector backbone is 15.5 kb in length, while the spectinomycin gene is restricted in the lower band. The 1Kb Ladder is represented by (L). (1) denotes KCS6 that has not been digested. (2) depicts the breakdown of KCS6.

Quantification of wax content

To cultivate the plants for seed collection, move the tray to a greenhouse or growth chamber. To confirm the existence of transgenic plants, the wild type and CRISPR mutants were compared. Leaf DNA was extracted using the CTAB method. A normal or wild-type plant was used as a negative control. To verify the plant's transformation, q/RTPCR was used. Wax quantification of both the wild type and CRISPR/Cas9 KCS6-mutant lines was done. It was observed that there was a significant difference in wax quantification between the wild-type and mutant lines. The percentage reduction in wax of the KCS6 knockout Arabidopsis plant ranged from 41% to 51% when comparing 10 wild-type and 10 mutant plants.

Table 2. Percentage of wax in wild-type and mutant lines

Wild type *Arabidopsis*	**KCS6 Mutant line**	**Percentage reduction**
Wax (µgcm²)	Wax (µgcm²)	
3	1.35	45%
2.50	1.23	49%
2.80	1.46	52%
2.77	1.14	41%
2.44	1.37	56%
2.91	1.37	47%
2.56	1.23	48%
2.88	1.53	53%
2.90	1.43	49%
2.75	1.41	51%

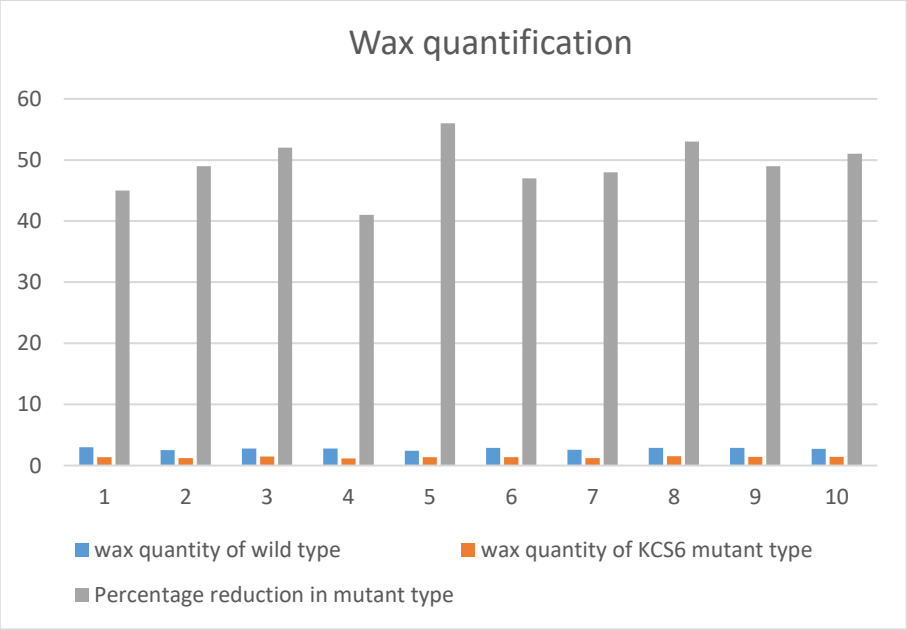

Figure 4. This figure shows the wax quantity of kcs6 in wild-type and mutant types.

The results presented in Table 2 clearly demonstrate that mutation of KCS6 significantly reduced cuticular wax accumulation in Arabidopsis thaliana. The cuticular wax content of wild-type plants ranged from 2.44 to 3.00 µg cm^{-2}. In contrast, the kcs6 mutant lines contained only 1.14–1.53 µg cm^{-2}, representing a reduction of 41–56%, with an average reduction of approximately 49%. The consistent reduction observed in all biological replicates indicates that KCS6 is an essential component of the cuticular wax biosynthetic pathway. This finding is consistent with the study of Huang et al (2022), who demonstrated that KCS6 is the principal β-ketoacyl-CoA synthase

responsible for elongating C26–C28 very-long-chain fatty acids (VLCFAs), which are the major precursors required for cuticular wax biosynthesis.

The biochemical function of KCS6 can explain the reduction in wax accumulation within the fatty acid elongase (FAE) complex. KCS6 catalyzes the first and rate-limiting condensation reaction during VLCFA elongation in the endoplasmic reticulum. Disruption of KCS6 therefore limits the production of VLCFAs that are subsequently converted into aldehydes, alkanes, primary alcohols, secondary alcohols, ketones, and wax esters, resulting in a substantial decline in total cuticular wax. Huang et al (2022) further showed that KCS6 functions together with CER2-LIKE proteins to extend fatty acid chain lengths beyond C28, demonstrating that KCS6 is indispensable for producing the predominant wax components deposited on the aerial surfaces of Arabidopsis. Similarly, Batsale et al (2023) highlighted that KCS enzymes constitute the substrate-specific component of the FAE complex and largely determine the composition and quantity of VLCFAs synthesized in plant epidermal tissues.

Although wax accumulation was reduced by approximately one-half in the kcs6 mutant, detectable amounts of wax remained, suggesting that KCS6 is not the sole enzyme responsible for VLCFA elongation. Instead, the remaining wax likely reflects functional redundancy among members of the KCS gene family. Huang et al (2022) demonstrated that KCS5 possesses overlapping substrate specificity with KCS6 and partially compensates for the loss of KCS6 function during wax biosynthesis. In addition, Luo et al (2024) reported that KCS19 also contributes to VLCFA elongation and cuticular wax formation while improving tolerance to drought and salt stress. These findings indicate that wax biosynthesis is regulated by a coordinated network of KCS enzymes rather than by a single elongase, explaining why the kcs6 single mutant retains approximately 50% of the wild-type wax content.

The reduction in cuticular wax observed in the kcs6 mutant is expected to affect the protective properties of the plant cuticle. Cuticular wax forms the outermost hydrophobic barrier of aerial tissues and functions to minimize non-stomatal water loss, prevent excessive cuticle permeability, protect against ultraviolet radiation, and reduce pathogen penetration. Consequently, plants with reduced wax accumulation generally exhibit increased cuticular transpiration and decreased tolerance to drought stress. Recent transcriptomic analyses by Urano et al (2024) demonstrated that drought stress strongly induces the expression of KCS6, CER1, CER26-LIKE, and other wax biosynthetic genes, resulting in increased wax deposition as an adaptive mechanism to reduce water loss. Likewise, the comprehensive review by Marques & Hu, (2024) concluded that enhanced wax biosynthesis is one of the most conserved adaptive responses of terrestrial plants exposed to water deficit, heat stress, and other abiotic stresses. Therefore, the nearly 49% reduction in wax content observed in the present study strongly suggests that the kcs6 mutant would possess a less effective cuticular barrier and consequently exhibit lower drought tolerance than wild-type plants.

The relatively small variation in wax reduction among biological replicates (41–56%) is likely attributable to normal biological variation and minor technical differences during wax extraction and quantification. Nevertheless, the reduction was consistently observed in every mutant line, indicating that the phenotype is directly associated with disruption of KCS6 rather than experimental variability. Similar reproducibility has been reported in previous studies investigating wax-deficient

mutants, where independent mutant lines consistently exhibited reduced wax accumulation despite minor differences in absolute wax quantities (Huang et al., 2022; Batsale et al., 2023).

Overall, the results presented in Table 2 confirm that KCS6 plays a pivotal role in cuticular wax biosynthesis in Arabidopsis thaliana. Mutation of KCS6 reduced wax accumulation by approximately 49%, confirming that this enzyme is a major determinant of VLCFA elongation and cuticular wax production. However, the persistence of residual wax in the mutant indicates that other members of the KCS family partially compensate for the loss of KCS6 activity. Collectively, these findings are consistent with recent molecular studies demonstrating that cuticular wax biosynthesis is regulated through coordinated interactions among KCS enzymes, CER2-LIKE proteins, and drought-responsive transcriptional regulators that together maintain cuticle integrity and enhance plant adaptation to environmental stress (Huang et al., 2022; Batsale et al., 2023; Luo et al., 2024; Urano et al., 2024; Marques & Hu, 2024).

CONCLUSION

This study demonstrated that CRISPR/Cas9-mediated knockout of the KCS6 gene significantly reduced cuticular wax accumulation in Arabidopsis thaliana, confirming the essential role of KCS6 in cuticular wax biosynthesis. The kcs6 mutant lines exhibited an average reduction of approximately 49% in wax content compared with wild-type plants, indicating that disruption of KCS6 impairs the elongation of very-long-chain fatty acids required for wax formation. Although residual wax was detected in the mutant lines, suggesting partial functional redundancy among members of the KCS gene family, the consistent reduction in wax accumulation highlights the predominant contribution of KCS6 to cuticle development. Given the well-established role of cuticular wax in limiting non-stomatal water loss, these findings further suggest that loss of KCS6 may compromise drought tolerance by weakening the protective function of the cuticle.

Despite these findings, this study has several limitations. It was conducted only in the model plant Arabidopsis thaliana under controlled growth conditions, which may limit the direct applicability of the results to crop species under field conditions. Future studies should validate the function of KCS6 homologs in economically important crops using genome-editing approaches and evaluate their performance under natural drought environments.

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