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Characterization of Kcs6 in wax biosynthesis and drought tolerance in *Arabidopsis thaliana*: Insights from Crispr/Cas9 gene knockout studies

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ABSTRACT

3-ketoacyl-CoA synthases (KCSs) are key enzymes of the fatty acid elongation (FAE) complex and are responsible for the production of very long-chain fatty acids (VLCFAs). Among them, KCS5 and KCS6 are known to participate in the elongation of C26 to C28 fatty acids and in cuticular wax biosynthesis. Drought is a major abiotic stress that reduces crop productivity and contributes to food insecurity. Plants are also exposed to several biotic and abiotic stresses, including pathogens, salinity, temperature extremes, and water deficiency. Arabidopsis thaliana is widely used as a model plant for investigating drought-response mechanisms. This study evaluated the role of KCS6 in drought tolerance and wax formation. The KCS6 gene was disrupted using CRISPR/Cas9 genome editing with a designed guide RNA targeting the gene. The construct was introduced into Agrobacterium and subsequently transformed into Arabidopsis using the floral-dip method. Transformation and mutation were confirmed through DNA extraction and PCR analysis. Cuticular wax content was then compared between wild-type plants and KCS6 knockout lines using chloroform extraction. Ten wild-type and ten mutant plants were evaluated. The KCS6 knockout plants showed a 41–51% reduction in wax content compared with the wild type. These results demonstrate that KCS6 contributes substantially to fatty-acid precursor formation and cuticular wax biosynthesis. Loss of KCS6 reduces wax accumulation and may consequently weaken the ability of Arabidopsis plants to tolerate drought and other abiotic stresses.

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INTRODUCTION

Drought is one of the major causes of malnutrition, hunger, and undernourishment affects agricultural productivity globally and regionally (Mansoor et al., 2022). Due to global warming drought has become a problem in agricultural productivity. There are many physiological and biochemical pathways that are affected by drought stress. Water accounts for 80–95% of the plant body's fresh biomass and plays an important role in a variety of physiological activities such as plant growth development, and metabolism (Wu et al., 2022). Arabidopsis thaliana is a tiny rosette plant that grows as a biennial or cold weather annual, part of the Brassicaceae taxonomic family (Franco-Navarro et al., 2025). Because Arabidopsis thaliana is a typical glycophyte and many other xerophytes

or desiccation-tolerant plants contain drought-resistant systems analogous to glycophyte, it is the most often used model organism for studying plant drought tolerance. Arabidopsis, like other blooming plants, dehydrates and retains its offspring for long periods at room temperature (Lei, Meng, Wang, Li, & Zhu, 2026). Combined with a newly designed method for producing gene knockout lines, has led (Mohamed, Khan, Basit, & Nutrition, 2024) many fundamental biologists to believe that Arabidopsis is the best model system for basic study in multicellular eukaryotic biology (Yaschenko, Alonso, & Stepanova, 2025). Deficiency of water and saline stress resistance in Arabidopsis plants improved after administration with the non-protein aminobutyric acid, which is reported to generate plant resistance to various diseases (Decsi et al., 2024).

CRISPR/Cas9 system has been widely used in knockout of an individual gene in many plant species. The major reason is because of its gene editing and repair mechanisms (Saini et al., 2023). The most commonly used CRISPR/Cas9 systems usually cut the double-stranded DNA and generate a double stand break (Rengasamy et al., 2024). Currently, Agrobacterium-mediated CRISPR/Cas9 genome editing is the most commonly used delivery method; for the majority of plant species, this method can achieve a high efficiency compared with other delivery methods (Mohamed et al., 2024). In model and agricultural plants, multiplex genome editing has been efficiently used. A large number of sequence-specific nucleases have made precise gene editing possible, enabling the development of new crop types that are adapted to changing climates (Chavhan et al., 2025).

Plants developed structural traits that allowed them to adapt to challenging environmental conditions through the secretion of waxy substances onto their surface (Xu, Wang, Huang, Tao, & Guo, 2025). The presence of waxes on the surface of plants serves to protect them from desiccation, UV light and frost damage, and acts as a barrier to combat diseases and regulate plant-insect interactions (Trivedi et al., 2026). Wax is made up of with very long chains fatty acid, known as VLCFAs, and their derivatives. Wax production begins in the plastid, where long-chain fatty acids (LCFAs) are synthesized by a fatty acid synthase (FAS) (Zhao et al., 2026). A fatty acid elongase (FAE) complex transports these synthesized products to the endoplasmic reticulum (ER), where it elongates them into very-long-chain fatty acids (VLCFAs). The elongation products go through more changes that create primary alcohols, esters, aldehydes, alkanes, secondary alcohols, and ketones (Li et al., 2022). VLCFAs are essential in the synthesis of cuticular wax throughout the wax manufacturing process (Zhukov & Popov, 2022). The exact makeup of the cuticle composition depending on the developmental phase, environmental factors species of plant, and tissues. Plant cuticular waxes are complex combinations of very long-chain fatty acids (VLCFAs). and their derivatives, including aldehydes, alkanes, primary and secondary alcohols, esters, and ketones, that can be fragmented using organic solvent (Han, Lu, Zhao, Ali, & Jiang, 2026). A fatty acid elongation complex (FAE), which includes 3-ketoacyl-CoA synthases (KCSs), catalyses these reactions (Chen et al., 2024).

KCSs are typically found in different species, and in Arabidopsis, there are 21 KCS genes come of these KCSs have been found to be specifically for the synthesizing VLCFAs with a particular chain length, offering a variety of substrates for sphingolipids, membrane lipids during the expansion from VLCFAs (very long chain fatty acids) C26 to C28 acids during wax formation, the KCS6 gene, also known as CER6, which has activity towards C22, C24, C26, and C28, plays a dominating role (Joubès et al., 2008; Quist et al., 2009). Additionally, KCS6 catalyses the stretching of C and C3 but it needs

the cofactors CER2 and CER26 to do so (Guo et al., 2016). Enhancing agriculturally crucial qualities in crops to enhanced resilience to abiotic and biotic stressors has been an ongoing global endeavor for a very long time (Batsale et al., 2023). It is essential to adopt efficient practices and cutting-edge technologies to preserve agricultural productivity while reducing the impacts of global warming and climate change (Huai et al., 2020). In this study, we assessed the impact KCS6 mutation on the production of wax in response to drought. The role of KCS6 in facilitating the wax production in Arabidopsis, as well as the combined impact of inhibiting genes on wax formation under regular or water-deficient circumstances are examined.

METHOD

Plant Materials and stress condition

Seeds of Arabidopsis thaliana were used in this study acquired from Cotton Biotechnology Laboratory, University of Agriculture Faisalabad and cultivated in a growth room under controlled conditions. Seeds grows on Murashige and skoog MS plates and place these in growing chamber set to 22°C. All well-germinated seedlings were transplanted to new pots two weeks after planting. Photoperiods (8 hours of darkness and 16 hours of light) and the optimal temperature (22°C) were observed. Three 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 KCS6vector and plasmid isolation

To investigate the activity of KCS6 Bacterial strain GV3101 was used in our study. The KCS6 vector was constructed, which consists of Plant Codon-Optimized Cas9, which is triggered by the 35S promoter of the Cauliflower Mosaic Virus, guarantees effective expression in plants (Gupta et al., 2023). The HindIII sites on either side of the 1.2 kb Spectinomycin Resistance Gene facilitate cell selection and possible removal. Essential components such as an origin of replication, selectable markers (such as genes for antibiotic resistance), a Multiple Cloning Site (MCS), and BsaI recognition sites for accurate cloning are all included in the 16.5 kb Vector Backbone (Fauser et al., 2026). 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 are incubated in a shaker (180 rpm) overnight at 37°C. Alkaline lysis solutions were used to extract the plasmid DNA. It was determined whether the plasmid DNAs were pure using an agarose gel. Plasmid DNA was extracted from small-scale bacterial colonies (Secgin, Kavas, Yildirim, & Forestry, 2021).

Preparation of Chemically Competent Cells of Agrobacterium Reagents

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

two hours in a 15 ml Corning tube. Centrifuged for 2 minutes at 5000 rpm. From 6–200 titers of medium were used to resuscitate the cells. Seven LB agar plates containing cells received the addition of Rifampicin and Gentamycin. 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 have enough water. 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. When secondary irradiance (just at flower opening) developed before floral emergence altered it. We performed that by vigorously shaking 5 ml of the agrobacterium cultured in media containing related antibiotics, such as hygromycin, rifampicin, gentamycin, and kanamycin, overnight at 28 °C. 1.2 to 1.5 was the O.D. Following overnight shaking, we spun it and then dissolved it in 2 ml of agro filtration medium. Once more, the O.D. was between 0.8 and 1.2. I dipped the flowers in it, giving it 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 wax

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; determine the average of these leaves for the correct wax content.

RESULTS AND DISCUSSION

Experimental procedure

Ligation of sgRNA into KCS6 vector

The annealed oligoes of gDNA with BsaI restriction site based sticky ends were ligated with the gv301 vector's gel eluted backbone. The GV301-sgRNA-Cas9 construct was ready to be transformed into a full plant expression vector.

Table 1. KCS6 Vector restriction confirmation with eestriction with enzyme Hindi-III (Doul 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

Transformation of constructs into Agrobacterium cells

After colony PCR, confirmed constructs were transformed into the Agrobacterium GV-3101 strain. The constructions were chosen on LB media containing 100 bp, 400 bp, and 500 bp. 1, 2, 3, 4, and 5 Colony PCR for ligation confirmation with 52 rifampicin, kanamycin, and gentamycin as mentioned in figure. After that, the constructions were streaked in LB plates for miniprep confirmation.

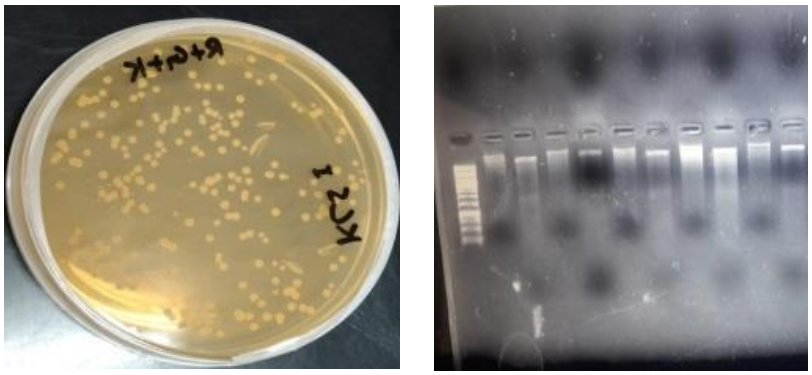


Figure 1. Agrobacterium GV-3101 was used to transform the confirmed constructs, which were then chosen on LB plates using antibiotics and confirmed by colony PCR and miniprep.

Confirmation of KCS6 Plasmid in Agrobacterium

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

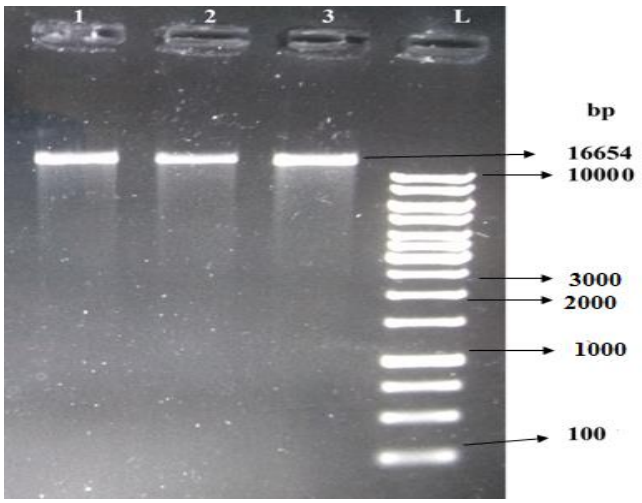


Figure 2. KCS6 plasmid confirmation on 1% agarose gel, 1, 2, and 3 are wells containing plasmid from distinct colonies of transformed agrobacterium cells, and L is the 1kb Ladder.

Confirmation of Restriction Digestion of KCS6

KCS6 plant expression vector with plant codon optimized Cas9 and 35S promoter was obtained from Addgene. The plasmid contains a 1.2kb spectinomycin gene with hindiIII restriction sites. KCS6 was digested using the BsaI(BsaI is 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.

This enzyme flanked the streptomycin resistance gene in KCS6 plasmid. The vector backbone is 15.5 kb in length, while the spectinomycin gene is restrained 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.

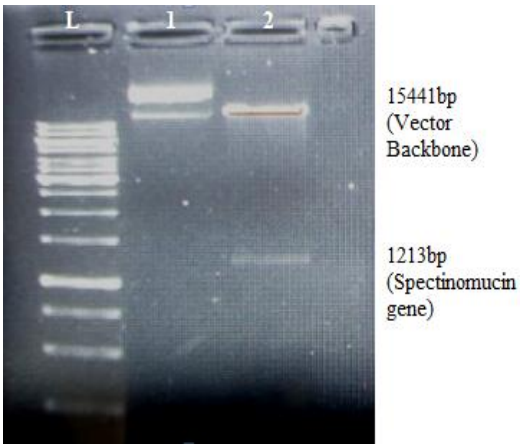


Figure 3. Confirmation of KCS6 restriction with HindiIII**.**

Germination of Arabidopsis seeds

Most seed lines germinate after 3–5 days of sowing when given constant sunshine, sufficient watering, and a healthy diet. Arabidopsis completes its life cycle in 8-12 weeks from germination to harvesting.

Quantification of wax content

Once their real leaves appear, transfer prospective transformants to a new selection plate. To confirm that they are genuine transgenic lines, let them grow for an additional one to two weeks (Ontiveros-Cisneros, Moss, Van Moerkercke, & Van Aken, 2022). Plant the seedlings in soil that has been wet with water, and then wrap the tray in plastic to maintain a high humidity level for two days (Jamison, Pandey, de Jesus Morais, Cardoso, & Garcia, 2025). 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 (Tang et al., 2022). 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 wild type and CRISPR/Cas9 KCS6-mutant line were done (Negin et al., 2024). It was observed that there was a significant difference of wax quantification in wild type and mutant line (Luo et al., 2024). The percentage reduction in wax of KCS6 knockout of Arabidopsis plant ranges (41%-51%) by comparing 10 wild type and 10 mutant plants.

Table 2. Percentage of quantification of wax 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%

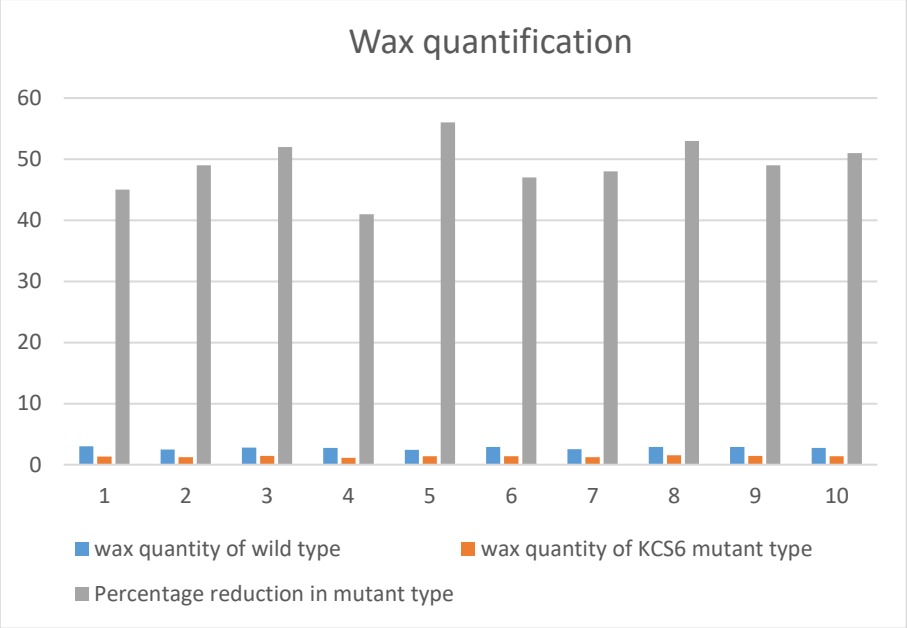


Figure 4. This figure shows the wax quantity of kcs6 in wild and mutant type. The red lines show wax quantity in mutant and blue lines exhibit wax quantity in wild type. The green lines indicate wax quantity which decreases gradually from 41 to 51%

KCSs catalyze the elongation of VLCFAs, supplying substrates for lipids with different expression patterns, such as sphingolipids, surface lipids, membrane lipids, and storage lipids (Joubès et al., 2008). Certain members of these KCSs, such as KCS6, KCS2, KCS20, and KCS9, have been linked to wax synthesis (Kim et al., 2013). These members are widely expressed in the epidermis, which is also the location of a large concentration of wax-related genes. The involvement of KCS6 in wax production was hypothesized because the sequences of the KCS6 coding protein and KCS5 are highly similar (Lewandowska, Keyl, & Feussner, 2020). It expresses itself specifically in the epidermis, the endoplasmic reticulum, where the wax components were made, is where their coding protein is located (Sun et al., 2025). In this work, we found that the KCS5 mutation has a clear impact on the wax content of rosette leaves because it reduces the amount of alkanes, which are the main constituents and imply that KCS6 is involved in the foliar in Arabidopsis (Lian, Gao, Jiang, Liu, & Li, 2021).

While KCS6 is involved in the manufacture of wax, the kcs5 mutant's wax phenotype is less pronounced than that of KCS6 mutants (Jiang et al., 2025). Compared to KCS6, our wax phenotypic research clearly shows that KCS5 is mostly irrelevant to the synthesis of wax (Huang et al., 2022). Mutant leaves and flowers show a drop of 32% and 86%, respectively, in wax levels of KCs6. While suppression of KCS6 activity resulted in a considerable reduction in stem wax quantities, which dramatically reduced to 13% that of the wild type, KCS6 mutation has no influence on stem wax synthesis. It is clear that with the kcs6-1 mutant, leaves are losing mass at a slower rate than other organs (Song et al., 2023). Since C29 alkane is considerably reduced in KCS6 rosette leaves, KCS6 appears to be more crucial in this mechanism (Weidenbach et al., 2015).

Several genes encode KCS enzymes. The first KCS family gene to be cloned from Arabidopsis was FAE1, which is only expressed in seeds. It's encoded protein catalyses the production of C20 and C22 fatty acids as well as storage lipids (Joubès et al., 2008). In this study, we show that the

Agrobacterium tumefaciens-delivered Cas9/sgRNA system is very functional when applied to Arabidopsis via the floral dip technique. Wax crystals in the stems and siliques of Arabidopsis KCS6 mutants are essentially virtually undetectable because of KCS6 function being lost (Huang et al., 2022). By contrasting 10 wild type and 10 mutant plants, the percentage reduction in wax in KCS6 knockout Arabidopsis plants ranged from 41% to 51%. Disrupting the KCS6 gene in Arabidopsis, it was possible to determine KCS6's crucial function in cuticle wax formation and how it affects drought tolerance (Liu et al., 2026). The outcomes increased our understanding of the decline in cuticular wax in Arabidopsis (Lv et al., 2025).

CONCLUSION

Utilization of CRISPR technology to knockout the KCS6 gene or eliminate its expression, allowing characterizing the role of KCS6 gene in wax synthesis. This study suggested that mutant KCS6 decrease the total amount of wax synthesis. This research uses the application of CRISPTR – CAS9 technology to the model plant *A. thaliana* that serves as a model for other plants with diverse genes involved in drought tolerance. Additionally, mutation in KCS6 gene blocks drought induced wax production. Implicating their key role in wax synthesis during drought.

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