ECERIFERUM 10 Encoding an Enoyl-CoA Reductase Plays a Crucial Role in Osmotolerance and Cuticular Wax Loading in Arabidopsis

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Acquired osmotolerance induced after salt stress is widespread across Arabidopsis thaliana (Arabidopsis) accessions (e.g., Bu-5). However, it remains unclear how this osmotolerance is established. Here, we isolated a mutant showing an acquired osmotolerance-defective phenotype (aod2) from an ion-beam-mutagenized M2 population of Bu-5. aod2 was impaired not only in acquired osmotolerance but also in osmo-shock, salt-shock, and long-term heat tolerances compared with Bu-5, and it displayed abnormal morphology, including small, wrinkled leaves, and zigzag-shaped stems. Genetic analyses of aod2 revealed that a 439-kbp region of chromosome 4 was translocated to chromosome 3 at the causal locus for the osmosensitive phenotype. The causal gene of the aod2 phenotype was identical to ECERIFERUM 10 (CER10), which encodes an enoyl-coenzyme A reductase that is involved in the elongation reactions of very-long-chain fatty acids (VLCFAs) for subsequent derivatization into cuticular waxes, storage lipids, and sphingolipids. The major components of the cuticular wax were accumulated in response to osmotic stress in both Bu-5 WT and aod2. However, less fatty acids, primary alcohols, and aldehydes with chain length ≥ C30 were accumulated in aod2. In addition, aod2 exhibited a dramatic reduction in the number of epicuticular wax crystals on its stems. Endoplasmic reticulum stress mediated by bZIP60 was increased in aod2 under osmotic stress. The only cer10 showed the most pronounced loss of epidermal cuticular wax and most osmosensitive phenotype among four Col-0-background cuticular wax-related mutants. Together, the present findings suggest that CER10/AOD2 plays a crucial role in Arabidopsis osmotolerance through VLCFA metabolism involved in cuticular wax formation and endocytic membrane trafficking.

Keywords: osmotolerance, cuticular wax, enoyl-CoA reductase, very-long-chain fatty acid, ER stress, Arabidopsis thaliana accession
INTRODUCTION

The plant cuticle is an extracellular lipid structure that covers the outer surface of land plants and protects the plant body from abiotic and biotic stresses (Javelle et al., 2011). Cuticle biosynthesis begins in the early stages of embryogenesis and is strictly coordinated with plant growth to supply constant cuticle deposition (Delude et al., 2016). The cuticle is divided into two domains: the underlying cuticular layer, which is a cutin-rich domain with embedded polysaccharides, and the overlying cuticle proper, which is enriched in waxes. A cutin matrix runs through both the cuticular layer and the cuticle proper. Cuticular waxes can be either embedded within the cutin matrix as intracuticular wax or accumulated on the cuticle surface as epicuticular wax crystals or films (Yeats and Rose, 2013; Ingram and Nawrath, 2017). Cuticular wax is a mixture of mostly aliphatic very-long-chain fatty acid (VLCFA; C20–34) derivatives that include alkanes, aldehydes, primary and secondary alcohols, ketones, and esters (Yeats and Rose, 2013). The parent VLCFAs are produced by the activities of four endoplasmic reticulum (ER)-bound enzymes that together form a fatty acid elongase (FAE) complex, and the biosynthesis occurs via the following four successive reactions that together form one reaction cycle: (i) condensation of C18-CoA and malonyl-CoA to form 3-ketoacyl-CoA (mediated by 3-ketoacyl-CoA synthase; KCS); (ii) reduction of 3-ketoacyl-CoA to 3-hydroxy-CoA (mediated by ketoacyl-CoA reductase; KCR); (iii) dehydration of 3-hydroxyacyl-CoA to enoyl acyl-CoA (mediated by 3-hydroxyacyl-CoA dehydratase; HCD); and (iv) reduction of enoyl acyl-CoA to form a saturated fatty acyl-CoA product (mediated by enoyl-CoA reductase; ECR). The reaction cycle synthesizes a two-carbon longer acyl-CoA and can be repeated to yield VLCFAs with various chain lengths ranging from C20 up to C38 or more (Batsale et al., 2021). These VLCFAs are then derivatized and subsequently incorporated into the cuticle as cuticular waxes, or converted to suberin, another plant surface barrier on the root endodermis, to triacylglycerol storage lipids, or to membrane lipids such as phospholipids or sphingolipids (Kim et al., 2013; Batsale et al., 2021).

In A. thaliana (Arabidopsis), several studies have identified genes encoding the enzymes that form the FAE complex. There are 20 KCS-encoding genes in the Arabidopsis genome, among which ECERIFERUM 6 (CER6)/KCS6/CUTICULAR 1 (CUT1) has been shown to play an important role in wax biosynthesis; a cer6/kcs6/cut1 mutant exhibits a severe waxless phenotype characterized by a decrease in longer wax compounds and accumulation of C24 and C26 derivatives (Millar et al., 1999; Fiebig et al., 2000). Complementation assays using yeast mutants have identified 3-KETOACYL-COA SYNTHASE 1 (KCS1) and PASTICCINO2 (PAS2) as Arabidopsis genes encoding a functional KCR enzyme and a functional HCD enzyme, respectively; complete loss of KCR1 or PAS2 activity leads to embryonic lethality, indicating that VLCFA biosynthesis is essential for embryonic development (Bach et al., 2008; Beaudoin et al., 2009). Similar complementation assays using yeast mutants have identified ECERIFERUM 10 (CER10) as an Arabidopsis gene that encodes a functional ECR enzyme (Kohlwein et al., 2001).

An Arabidopsis cer10 mutant is reported to show morphological abnormalities such as reduced size of aerial organs as well as a reduction of cuticular wax load and altered VLCFA composition of seed triacylglycerols and sphingolipids; in addition, the Golgi apparatus is larger and tends to form ring-like clusters, resulting in a possible defect in endocytic membrane transport (Zheng et al., 2005).

Arabidopsis plants subjected to drought, salt, or abscisic acid treatments exhibit a significant increase in the amounts of cuticular lipids in their leaves (Kosma et al., 2009). An R2R3-type MYB transcription factor, AtMYB49, has been shown to contribute to salt tolerance in Arabidopsis by increasing cutin deposition in the leaves (Zhang et al., 2020). Ectopic expression of Newhall navel orange GsKCS6, an ortholog of Arabidopsis KCS6, has been shown to increase the amount of VLCFAs in the cuticular wax on the stems and leaves of Arabidopsis plants and to improve drought and salt tolerances (Guo et al., 2020). These reports suggest a relationship between cuticular wax and drought or salt tolerance in Arabidopsis; however, studies are needed to clarify the relationship between FAE complex including ECR and abiotic stress tolerances, and between reduced cuticular wax levels and stress tolerances.

Natural genetic variation in Arabidopsis has facilitated identification of plant genes governing complex traits such as growth, flowering, and stress tolerance (Alonso-Blanco et al., 2009; Weigel, 2012). Previously, we found a wide variation in salt tolerance among Arabidopsis accessions; in addition, we found that while most salt-tolerant accessions also exhibited acquired osmotolerance after salt stress (e.g., Bu-5), others did not (e.g., Col-0; Katori et al., 2010; Ariga et al., 2017); however, little is known about which genes contribute to such acquired osmotolerance. Here, to understand more about the mechanism underlying acquired osmotolerance in Arabidopsis, we isolated and characterized a mutant showing an acquired osmotolerance-defective phenotype (aod2) from the Arabidopsis accession Bu-5.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis seeds (Bu-5 or Col-0) were sown on agar (1%, w/v) plates containing full-strength Murashige and Skoog (MS) medium containing a vitamin mixture (10 mg L\(^{-1}\) of myoinositol, 200 µg L\(^{-1}\) of glycine, 50 µg L\(^{-1}\) of nicotinic acid, 50 µg L\(^{-1}\) of pyridoxine hydrochloride, and 10 µg L\(^{-1}\) of thiamine hydrochloride; pH 5.7) and 1% sucrose. The plates were sealed with surgical tape and the seeds were stratified at 4 °C for 4–7 days before being transferred to a growth chamber (80 µmol photons m\(^{-2}\) s\(^{-1}\); 16 h/8 h light/dark cycle; 22°C) for germination and growth.

To obtain Arabidopsis mutants, Bu-5 seeds were irradiated in the azimuthally varying field cyclotron at the Japan Atomic Energy Agency (Takasaki, Japan). To select the appropriate dose, we irradiated the seeds with carbon ion beams in a dose range of 25–250 Gy and assessed plant development. Doses of 200 Gy or higher inhibited secondary leaf development or...
induced sterility. Therefore, we irradiated the seeds at 150 Gy in a single layer within a plastic bag.

Seeds of the following four Arabidopsis mutants were obtained from the Arabidopsis Biological Resource Center (Ohio State University): cer10 (SALK-088645), lcr (SALK-136833C), cer2 (SALK-127158), and cer5 (SALK-036776).

Stress Treatment for the Acquired Osmotolerance Assay

Seven-day-old seedlings grown on nylon mesh (990 μm) on an M&S agar plate were transferred with the underlying mesh (hereafter “mesh-transferred”) to a plate supplemented with 100 mM NaCl for 7 days. The seedlings were then mesh-transferred to a plate supplemented with 750 mM sorbitol for 15 days.

Abiotic Stress Assays

Ten-day-old seedlings grown on nylon mesh (990 μm) on an M&S agar plate were mesh-transferred to a plate supplemented with 200 mM NaCl for 8 days (salt-shock stress), 600 mM sorbitol for 21 days (osmo-shock stress), or 10 μm paraquat for 7 days (oxidative stress). For the long-term heat tolerance assay, the plates with 10-day-old seedlings were placed at 37°C for 4 days and then moved back to 22°C for 5 days. Chlorophyll content was determined as in (Porra et al., 1989).

RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction

Total RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR) analysis were performed as described in Isono et al. (2020). ACTIN2 was used as the internal standard for qRT-PCR. The primers used are listed in Supplementary Table S1.

Genetic Mapping of the Causative Gene of the aod2 Phenotype

aod2 was crossed with an osmo-tolerant accession, Pog-0, and the resulting F1 progeny were selfed to generate F2 populations. Genomic DNA was prepared from individual F2 plants with the recessive phenotype for use as PCR templates. The simple sequence length polymorphism markers listed in Supplementary Table S2 were used for mapping. The PCR conditions were as follows: (94°C for 2 min) 1 cycle, (94°C for 20 s, 52°C–55°C for 20 s, and 72°C for 20 s) 34 cycles, and (72°C for 2 min) 1 cycle. Microsatellites were fractionated in a 5%–7% agarose gel, and the recombination frequencies (%) were calculated from the band pattern.

DNA Library Construction and Sequencing of aod2 Mutant

DNA library construction and sequencing were performed as described in (Tsukimoto et al., 2021). The read data were submitted to the DNA Data Bank of Japan Read Archive (accession number DRA013622).

Detection of Mutations in the aod2 Mutant

Detection of mutations from the whole-genome sequencing data of the aod2 mutant was performed as described in Tsukimoto et al. (2021). For detection of the 439-kbp insertion from Chr. 4 into the causal region in the aod2 mutant, we designed the primer sets listed in Supplementary Table S3. Primer sets (i) and (ii) were designed to detect DNA bands only in the presence of the 439-kbp insertion, and primer set (iii) was designed to detect DNA bands only in the presence of deletion.

Plasmid Construction and Transformation

For complementation analysis, the genomic region of AOD2/CER10 (2.0-kb upstream of the ATG initiation codon and 1.0-kb downstream of the termination codon of Bu5) was amplified by PCR using the pRI909 CER10 primers (Supplementary Table S3) and then cloned into the binary vector pRI909. The constructs were introduced into Agrobacterium tumefaciens GV3101, which was used for plant transformation by the floral dip method. Transgenic plants were selected on M&S agar plates containing 200 μg ml−1 Claforan and 25 μg ml−1 kanamycin. Ten-day-old seedlings (T1 plants) were transferred to soil in pots.

Toluidine Blue Test

The aerial parts of 2-week-old seedlings were submerged in aqueous solution of 0.05% (w/v) toluidine blue (TB; Sigma, St Louis, MO, United States). After 20 min on a shaker set at 100 rpm, the TB solution was removed, and the plates were washed gently with water to remove excess TB from the plants. Next, the plants were homogenized in a 1.5-ml tube containing zirconia beads. Then, 200 μl of buffer [200 mM Tris–HCl (pH 8.0), 250 mM NaCl, and 25 mM EDTA] and 400 μl of ethanol was added, with vortex mixing, and plant debris was pelleted by centrifugation (15,000 rpm for 10 min). The supernatant was examined spectrophotometrically, and the amount of TB was determined from the absorbance at 630 nm (A630). The major peak of absorbance due to plant material (A435) was used for normalization. Relative levels of TB were calculated as the ratio of A630 to A435 (Tanaka et al., 2007).

Scanning Electron Microscopy

A 3-cm section from the base of the flower stem 2 weeks after bolting was used for observation of the epidermal surface. The stem was cut into 5–10 mm pieces and coated with Pt + Pd using an E102 ion sputtering system (Hitachi, Tokyo, Japan) and used for scanning electron microscopy (S4800, Hitachi).

Water Loss Assay

Leaves of 4-week-old plants grown in soil under normal growth conditions were detached and then left in ambient conditions. The weight was measured every 10 min for 1 h. The percentage decreases in fresh weight are expressed as percentage water loss.
Extraction of Cuticular Waxes and Gas Chromatography-Mass Spectrometry Analysis

Two-week-old Bu-5, aod2, and Col-0 seedlings grown on MS agar plates (control condition) and salt-acclimated for 1 week were mesh-transferred to MS agar plates containing 750 mM sorbitol for 21 days (osmo-stress condition). The cuticular wax of leaves (six biological replicates) was extracted by immersion in chloroform containing tricosanoic acid as an internal standard for 10 s. The solvent was evaporated in a steam of nitrogen. Free hydroxyl and carboxyl groups were silylated with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA+TMCS, Sigma-Aldrich, St. Louis, MO, United States) for 1 h at 80°C. The wax composition was analyzed by using a GC2020 gas chromatograph (Shimadzu Inc., Kyoto, Japan) with the injector in splitless mode and the following temperature program: increase to 80°C, 15°C per min to 200°C, 3°C per min to 300°C and hold for 10 min at 300°C. Mass spectrum data were obtained on a GCMS-QP2020NX mass spectrometer (Shimadzu) after impact ionization. The peaks were quantified by using the LabSolutions software (Shimadzu). The amount of each wax monomer was determined on the basis of the internal standard and was normalized by leaf surface area. Leaf surface area was calculated by using the ImageJ software (Schneider et al., 2012).

RESULTS

Isolation of aod2

Previously, we found that Arabidopsis accession Bu-5 showed acquired osmotolerance (Katori et al., 2010; Ariga et al., 2017). Using this accession, we screened 30,000 ion-beam-mutagenized seedlings for mutants showing an acquired osmotolerance-defective phenotype and subsequently obtained the mutant aod2. Compared with Bu-5, aod2 showed significantly lower osmotic tolerance to 750 mM sorbitol after pre-exposure to 100 mM NaCl for 7 days (Figures 1A, B). In addition, soil-grown aod2 plants displayed abnormal morphogenesis in all aerial parts compared with Bu-5 plants grown under the same conditions: the leaves of aod2 plants were narrower and smaller (Figure 2A); the flower stems were shorter and exhibited a zigzag-shaped appearance (Figure 2B); some stamens displayed a zigzag-shaped appearance (Figure 2B); and the leaf trichomes were shorter and crooked (Figure 2C).

Characterization of aod2

To investigate whether the phenotype of aod2 was specific to acquired osmotolerance, we evaluated the tolerance of aod2 to three other abiotic stresses—osmo-shock, salt-shock, and oxidative stress (paraquat). aod2 showed significantly lower osmo-shock and salt-shock tolerance (Figure 3A) but the same oxidative stress tolerance (Supplementary Figure S1) compared with Bu-5. When we compared the expression of the osmostress-responsive marker genes COR15A, KIN1, RAB18, and RD29A, we found that the mRNA accumulation levels of all four genes were comparable between Bu-5 and aod2 (Figure 3B), indicating that the weakened osmotic stress tolerance in the mutant was not due to defective transcriptional regulation. We also found that aod2 showed significantly greater sensitivity to long-term heat stress (37°C, 4 days) compared with Bu-5 (Figure 3C).

Identification of the Causal Gene of the aod2 Phenotype

To identify the locus responsible for the osmosensitive phenotype of aod2, we crossed aod2 with Pog-0, an accession with acquired osmotolerance (Ariga et al., 2017), and subjected the mutant to high-resolution chromosomal mapping analysis of the locus within a 226-kbp region on the short arm of chromosome 3 (Figure 4A). The sequence variations within this region in the aod2 genome were examined by whole-genome sequencing. There were no non-synonymous mutations in any of the genes within the 226-kbp region; however, there was a 439-kbp insertion whose sequence was identical to that of part of chromosome 4. To verify the insertion, we performed PCR-based genotyping using primers spanning chromosomes 3 and 4 and confirmed that the 439-kbp insertion disrupted genes At3G55340, At3G55350,
and At3G55360 (Supplementary Figure S2). To identify which of the disrupted genes was the causal gene of the osmosensitive phenotype \textit{aod2}, we performed complementation tests in which each of the three disrupted genes derived from Bu-5, with their regulatory region, was introduced into \textit{aod2}. Only the introduction of At3G55360 (\textit{aod2\_At3G55360}) restored acquired osmotolerance as in Bu-5, indicating that At3G55360 was the causal gene of the osmosensitive \textit{aod2} phenotype (Figure 4B). At3G55360 was identical to CER10, which encodes an ECR involved in the final stage of the VLCFA elongation reaction cycle that produces VLCFAs for subsequent derivatization to cuticular waxes, storage lipids, and sphingolipids. In Bu-5, the transcription level of \textit{CER10/AOD2} was increased by osmotic stress (Figure 4C).

**Content and Composition of the Cuticular Wax**

ECR is an enzyme that catalyzes the final step of VLCFA elongation, and VLCFAs are the precursors of all the aliphatic components of cuticular wax. Therefore, to investigate the biochemical basis for the observed morphological phenotypes in \textit{aod2}, we examined the wax components in the leaves of Bu-5, \textit{aod2}, and Col-0 (a salt-sensitive accession that does not exhibit acquired osmotolerance). First, we examined total wax content (composite of fatty acids, primary alcohols, aldehydes,
and alkanes) and found no differences between Bu-5 and aod2 under control or osmotic stress condition, while Col-0 had less wax content compared to Bu-5 and aod2 under osmotic stress condition (Figure 5A). When we stratified the wax components into fatty acids, primary alcohols, aldehydes, and alkanes, increases in each type were detected in both Bu-5 and aod2 under osmotic stress compared with control (Figure 5B). Stratifying by chain length, the amount of each wax component with chain length ≥ C28 in Col-0 was lower than that in Bu-5 under osmotic stress (Figure 5B). In addition, aod2 had lower amounts of fatty acids, primary alcohols, and aldehydes with chain length ≥ C30 compared with those in Bu-5. The amounts of C26 and C28 fatty acids were higher in aod2 than in Bu-5, although those of C28 primary alcohols and aldehydes were lower in aod2 than in Bu-5. The aod2 exhibited a decrease in C26–C32 fatty acids but an increase in C16 and C18 fatty acids compared to WT (Supplementary Figure S3). No significant difference in alkane content was observed between Bu-5, aod2, and Col-0. Together, these findings suggest that accumulation of cuticular wax components plays an important role in the Arabidopsis response to osmotic stress, and that CER10/AOD2 contributes to the biosynthesis of ≥ C26–C30 fatty acids, primary alcohols, and aldehydes.

**Wax Layer of aod2**
Absence of ECR activity results in a reduction of cuticular wax load in Arabidopsis (Zheng et al., 2005). We therefore examined the integrity of the surface wax layer of the cuticle in aod2 by using the toluidine-blue (TB) test, which was established for detection of cuticular defects in whole leaf; a deficient cuticle allows TB to permeate the epidermal surface (Tanaka et al., 2004). The true leaves of aod2 were significantly stained with TB, whereas those of Bu-5 were not (Figure 6A). To investigate the morphology of the surface wax layer, we observed the epicuticular wax crystals on the stems of Bu-5 and aod2 by scanning electron microscopy; there were many granular wax crystals on the surface of the Bu-5 stems, but much less on the aod2 stems (Figure 6B). The aod2_At3g55360 plants exhibited wax crystals on the surface of stems like Bu-5 WT plants (Supplementary Figure S4). These findings suggest that CER10/AOD2 plays an important role in the development of epicuticular wax
crystals. Although there are differences in the wax contents on the leaf surface as shown in Figure 5, there were no clear differences in leaf surface between WT and aod2 (Supplementary Figure S5).

**Water Loss From Detached aod2 Leaves**

We examined whether CER10/AOD2 affected the water loss by detaching the whole aerial part of plants and leaving them under ambient conditions. After 9 h under ambient conditions, the aod2 leaves were much more shriveled than the Bu-5 leaves (Figure 7A). In addition, the rate of water loss from aod2 leaves was significantly faster than that from Bu-5 leaves (Figure 7B). These findings indicate that CER10/AOD2 plays an important role in water retention of plants.

**Endoplasmic Reticulum Stress in aod2 Under Osmotic Stress**

In a Col-0-background cer10 mutant, the loss of CER10 has been shown to decrease the amounts of membrane sphingolipids and to result in the formation of the Golgi stacks that display...
aggregate, ring-like structures, suggesting that CER10 plays a role in endocytic membrane trafficking (Zheng et al., 2005). Moreover, defective ER-to-Golgi transport has been shown to impair the salt and long-term heat tolerances in Arabidopsis (Isono et al., 2020). To investigate the differences in the ER stress response in Bu-5 and aod2 under normal and osmotic stress conditions, we examined the transcript levels of bZIP17, bZIP28, and bZIP60, which are major activators of the ER stress response (Howell, 2013). The transcript level of bZIP60 in aod2 was significantly higher than that in Bu-5 under osmotic stress (Figure 8A). Moreover, under osmotic stress conditions, the transcript levels of the two genes regulated by bZIP60 (i.e., SAR1A and SEC31A) were also significantly higher in aod2 than in Bu-5 (Figure 8B).

**Osmotolerance of Mutants Defective in Wax Biosynthesis**

There has been no comparative study of which genes contributing to cuticular wax load play roles in osmotic tolerance. Therefore, we evaluated the osmotolerance of Col-0 and four Col-0-background mutants defective in cuticular wax load: cer10, cer2, cer5, and leaf curling responsiveness (lcr; Millar et al., 1999; Pighin et al., 2004; Voisin et al., 2009; Haslam and Kunst, 2013, 2020; Pascal et al., 2013). Of these four mutants, only cer10 was significantly sensitive to osmotic stress than Col-0 (Figure 9A). To investigate the relationship between the osmotic tolerance of the mutants and wax biosynthesis, the TB-test and observation of epicuticular wax crystals on the stem of the mutants by scanning electron microscopy were conducted. Of the four mutants, cer10 and cer5 were stained with significantly more TB compared with Col-0; cer10 was stained much more than cer5 (Figure 9B). Consistent with the results of the TB-test, cer10, followed by cer5, displayed a marked decrease in the number of epicuticular wax crystals on the epidermal surface of the stem compared with that in Col-0 (Figure 9C). Although cer2 and cer5 have been reported to show reduced amounts of epidermal wax crystals on the stem surface (Xia et al., 1996; Jenks et al., 2002), we did not observe obvious reductions in the present study. These findings suggest that osmotolerance in Arabidopsis is correlated with wax biosynthesis, and that CER10 plays a crucial role in both osmotolerance and wax loading in Arabidopsis.

**DISCUSSION**

Here, we isolated a new Arabidopsis mutant, aod2, showing an osmotolerance-defective phenotype. The causal gene of the phenotype is identical to CER10, which encodes an ECR essential for the biosynthesis of VLCFAs. In Arabidopsis, VLCFAs are accumulated in response to osmotic stress, suggesting that VLCFA derivatives, which include components of the cuticular wax and sphingolipids, are an important part of osmotolerance.

It is reported that a defect in CER10 leads to Golgi aggregation (Zheng et al., 2005). In aod2, the expression levels of bZIP60 and the two genes it regulates (SAR1A and SEC31A), which are involved in the ER stress response, were increased under osmotic stress compared to the levels in Bu-5. These results suggest that ER stress is enhanced under osmotic stress in aod2. It is known that enhancement of ER stress impairs salt and long-term heat stress tolerances in Arabidopsis (Isono et al., 2020). In Arabidopsis, unsaturated aliphatic components account for about 60% of total stem cutin, with C18:2 dioic acid being the predominant unsaturated component (Bonaventure et al., 2004). It is known that FATTY ACID DESATURASE2 (FAD2) affected cutin monomer composition, and the fad2 mutation causes a 2-fold reduction in the levels of C18:2 dioic acids. The fad2 mutant exhibited hypersensitivity to tunicamycin, a chemical inducer of ER stress, suggesting that cutin composition and membrane lipid polyunsaturation are involved in ER stress tolerance in Arabidopsis (Nguyen et al., 2019). Very recently, we identified CYP76A5 encoding a cytochrome P450 monoxygenase known as KLU that can confer acquired osmotolerance and heat tolerance to Col-0 WT plants (Kajino et al., 2022). Contrary to the aod2 mutant, the transgenic Col-0 plants overexpressing AtKLU (AtKLUox) developed denser cuticular wax and accumulated higher levels of VLCFAs than WT plants. Furthermore, ER stress induced by osmotic or heat stress

FIGURE 7 | Water loss from detached acquired osmotolerance-defective (aod2) mutant leaves. (A) Leaves of 4-week-old plants grown in soil under normal growth conditions were detached (normal condition) and then left in ambient conditions for 9h (drought). (B) Percentage decreases of fresh weight are expressed as percentage water loss. Differences between Bu-5 (WT) and aod2 were analyzed by Student’s t-test (mean ± se, n = 3, **p < 0.01, ***p < 0.001).
was reduced in AtKLUox plants compared to WT plants (Kajino et al., 2022). Taken together, the hypersensitivity of aod2 mutant to osmotic and long-term heat stresses may be related not only to a decrease in cuticular wax content but also to enhancement of ER stress.

Most intracellular VLCFAs in plant have less than 26 carbon atoms (≤C26), whereas plant cuticular VLCFAs and their derivatives generally have about 30 carbon atoms (Hegebarth et al., 2016). We showed that the major components of the cuticular wax, which include fatty acids, primary alcohols, aldehydes, and alkanes longer than C26, were increased in response to osmotic stress after salt acclimation treatment, suggesting that accumulation of wax components plays an important role in osmotolerance in Arabidopsis Bu-5. Although there was no difference in total wax content between Bu-5 and aod2, in the mutant, the amounts of >C30 fatty acids and >C28 primary alcohols and aldehydes were reduced, indicating that these compounds contribute to osmotolerance. The amounts of C26 and C28 fatty acids were notably increased in aod2 compared with those in Bu-5, suggesting that CER10/AOD2 plays a role in the biosynthesis of fatty acids longer than C30. Although the amounts of the individual wax components were reduced in aod2 compared with those in Bu-5, each wax component was still detected, suggesting the

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**FIGURE 8** | Effect of the sloh4 mutation on expression of endoplasmic reticulum stress-related genes under osmotic stress. (A) Transcript levels of bZIP17, bZIP28, and bZIP60 in Bu-5 (WT) and aod2 plants under normal and acquired osmotic stress (100 mM NaCl for 7 days and subsequent 750 mM sorbitol for 10 days) conditions. (B) Expression of the target genes for the transcription factor bZIP60 in WT and aod2 under normal and osmotic stress conditions; expression levels were determined by quantitative real-time polymerase chain reaction relative to those of Actin2 (mean ± SE, n = 3). Differences between WT and aod2 were analyzed by Student’s t-test. *p < 0.05; **p < 0.01; ***p < 0.001.
However, we found that

\[ \text{Equation} \]

The Arabidopsis mutant \( \text{aod2} \) lacks \( \text{CER10} \) and shows not only impaired acquired osmrotolerance, but also impaired osmo-shock, salt-shock, and long-term heat tolerances. \( \text{CER10} \) encodes an ECR involved in VLCFA biosynthesis. In \( \text{aod2} \), the amounts of major wax components were decreased, and ER stress mediated by \( \text{bZIP60} \) was enhanced under osmotic stress compared with those in \( \text{Bu-5} \), indicating that \( \text{CER10} \) plays a crucial role in these abiotic stress tolerances mediated by \( \text{bZIP60} \).

When we evaluated the osmotolerance of various mutants lacking in cuticular wax load, we found that the degree of TB staining and the reduction of wax crystals on the epidermal surface were correlated with osmotolerance. However, we also found that the osmotolerance of the \( \text{lcr} \) mutant, which is defective in the amount of cutin but not in the amount of wax (Wellesen et al., 2001), was similar to that of \( \text{Bu-5} \). Loss-of-function \( \text{myb49} \) mutants and chimeric \( \text{AtMYB49-SRDX} \)-overexpressing \( \text{SRDX49} \) transcriptional repressor plants exhibit decreased amounts of cutin and hypersensitivity to salt stress, suggesting that the amount of cutin correlates with salt tolerance (Zhang et al., 2020). However, \( \text{AtMYB49} \) is involved not only in cutin content but also in the abscisic acid response via interaction with \( \text{AB15} \), and \( \text{SRDX49} \) plants show a reduced abscisic acid response (Zhang et al., 2020). Thus, the relationship between reduced cutin content and salt tolerance remains unclear. The osmotolerances of the \( \text{cer2} \) and \( \text{cer5} \) mutants were similar to that of \( \text{Bu-5} \). It is reported that the amount of alkanes is reduced in \( \text{cer2} \) compared with that in \( \text{Col-0} \), whereas the amounts of fatty acids, aldehydes, and primary alcohols remain unaltered (Pascal et al., 2013). However, we found that there was no difference in the amounts of alkanes in \( \text{Bu-5, aod2, and Col-0} \), whereas fatty acids, aldehydes, and primary alcohols were decreased in the osmosensitive accessions \( \text{aod2} \) and \( \text{Col-0} \) compared with the amounts in the osmotolerant accession \( \text{Bu-5} \). These findings suggest that alkanes may not have a significant effect on osmotolerance in \( \text{Arabidopsis} \). It has also been reported that the osmotolerance of \( \text{cer5} \) is not affected by the presence of \( \text{ABCG11} \), a homolog of \( \text{CER5} \) that also contributes to the extracellular transport of wax components (Pighin et al., 2004).

**CONCLUSION**

The \( \text{Arabidopsis} \) mutant \( \text{aod2} \) lacks \( \text{CER10} \) and shows not only impaired acquired osmrotolerance, but also impaired osmo-shock, salt-shock, and long-term heat tolerances. \( \text{CER10} \) encodes an ECR involved in VLCFA biosynthesis. In \( \text{aod2} \), the amounts of major wax components were decreased, and ER stress mediated by \( \text{bZIP60} \) was enhanced under osmotic stress compared with those in \( \text{Bu-5} \), indicating that \( \text{CER10} \) plays a crucial role in these abiotic stress tolerances via VLCFA metabolism.

**Figure 9** | Osmotolerance of mutants defective in wax biosynthesis. (A) Top: representative images showing osmo-shock tolerance in \( \text{Col-0} \) and \( \text{Col-0-background} \) \( \text{cer10, cer2, cer5, and lcr} \) mutants. Two-week-old seedlings were mesh-transferred to MS agar plates containing 600 mM sorbitol for 11 days. Bottom: Chlorophyll contents of the mutant plants shown in the top panel. (B) Toluidine blue (TB) test using \( \text{Col-0} \) and the \( \text{Col-0-background} \) mutants. TB uptake was examined spectrophotometrically by measuring absorbance at 630 nm (A630). The major peak of absorbance due to plant material (A435) was used for normalization. Relative levels of TB were calculated as the ratio of A630 to A435. (C) Scanning electron microscopy images of the surfaces of the stems of \( \text{Col-0} \) and the \( \text{Col-0-background} \) mutants. Bars = 100 μm. Data are presented as mean ± SE, n = 3. Letters at the top of columns are based on one-way ANOVA and Tukey’s test, p < 0.05.
which is required for cuticular wax synthesis and endocytic membrane trafficking. However, it remains unclear which VLCFA derivatives are critical for osmotolerance. Comparisons between osmotolerance and the amount of VLCFA derivatives in wax load-defective mutants, or screening for revertant mutants in which acquired osmotolerance is restored, should allow us to address this question.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

AUTHOR CONTRIBUTIONS

NF and TT initiated, conceived, and coordinated the project. TK isolated the aod2 mutant. NF identified the causal gene and performed physiological and biochemical analyses. HA and KT performed genome sequencing. NF and TK observed epicuticular wax crystals by scanning electron microscopy with YY. YO determined the wax components. TT wrote the manuscript with assistance from IY and YS. All authors contributed to the article and approved the submitted version.

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