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CAD1 contributes to osmotic tolerance in *Arabidopsis thaliana* by suppressing immune responses under osmotic stress

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ABSTRACT

Acquired osmotolerance induced by initial exposure to mild salt stress is widespread across *Arabidopsis thaliana* ecotypes, but the mechanism underlying it remains poorly understood. To clarify it, we isolated *acquired osmotolerance-deficient 1* (*aod1*), a mutant highly sensitive to osmotic stress, from ion-beam–irradiated seeds of Zu-0, an ecotype known for its remarkably high osmotolerance. *Aod1* showed growth inhibition with spotted necrotic lesions on the rosette leaves under normal growth conditions on soil. However, its tolerance to salt and oxidative stresses was similar to that of the wild type (WT). Genetic and genome sequencing analyses suggested that the gene causing *aod1* is identical to *CONSTITUTIVELY ACTIVATED CELL DEATH 1* (*CAD1*). Complementation with the WT *CAD1* gene restored the growth and osmotolerance of *aod1*, indicating that mutated *CAD1* is responsible for the observed phenotypes in *aod1*. Although *CAD1* is known to act as a negative regulator of immune response, transcript levels in the WT increased in response to osmotic stress. *Aod1* displayed enhanced immune response and cell death under normal growth conditions, whereas the expression profiles of osmotic response genes were comparable to those of the WT. These findings suggest that autoimmunity in *aod1* is detrimental to osmotolerance. Overall, our results suggest that *CAD1* negatively regulates immune responses under osmotic stress, contributing to osmotolerance in Arabidopsis.

1. Introduction

Drought-, salt-, or cold-induced osmotic stress inhibits plant growth. Plants can acquire stress tolerance after initial exposure to mild stress [1]. *Arabidopsis thaliana* accessions commonly acquire osmotolerance following salt stress [2]. Exposure of 7-day-old seedlings of some Arabidopsis accessions to 100 mM NaCl for 7 days induced osmotolerance to 750 mM sorbitol [2]. We have identified a nucleotide-binding leucine-rich repeat gene named *ACQUIRED OSMOTOLERANCE* (*ACQOS*) as the gene responsible for the acquired osmotolerance [3]. The protein interacts with PHYTOALEXIN DEFICIENT 4 (PAD4) and ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1) in the nucleus to activate the immune response [4]. ACQOS plays a positive role in bacterial resistance in the absence of osmotic stress but triggers detrimental autoimmunity under osmotic stress through EDS1 and PAD4, thereby compromising osmotolerance [3]. The detrimental autoimmunity induces programmed cell death (PCD) [5]. Accessions harboring functional *ACQOS* alleles (e.g., Col-0) do not acquire osmotolerance, whereas those with non-functional alleles (e.g., Zu-0 and Bu-5) acquire osmotolerance [3]. However, little is known about which genes contribute. To elucidate the mechanism, we screened mutants showing an acquired osmotolerance defective phenotype (*aod*) from ion-beam–mutagenized Zu-0 or Bu-5 seeds. Among the identified *aod* mutants, *aod2* carries a mutation in the *ECERIFERUM 10* (*CER10*) gene, which contributes to the elongation of very-long-chain fatty acids, substrates for cuticular wax synthesis [6]. *Aod6* harbors a mutation in the *CATION CALCIUM EXCHANGER 4* (*CCX4*) gene, encoding a calcium ion transporter localized in the Golgi apparatus [7], and *aod13* has a mutation in the *MAP KINASE PHOSPHATASE 1* (*MKP1*) gene, responsible for dephosphorylating MITOGEN-ACTIVATED PROTEIN KINASE 3/6 (MPK3/6) [8]. These findings suggest the importance of cuticular wax accumulation, Ca^{2+} transport via CCX4, and suppression of the immune response through MPK3/6 phosphorylation in acquired osmotolerance. *Aod6* and *aod13* showed an enhanced immune response under osmotic stress, suggesting the importance of suppressing the immune response in osmotolerance.

Lesion-mimetic mutants that constitutively misregulate cell death

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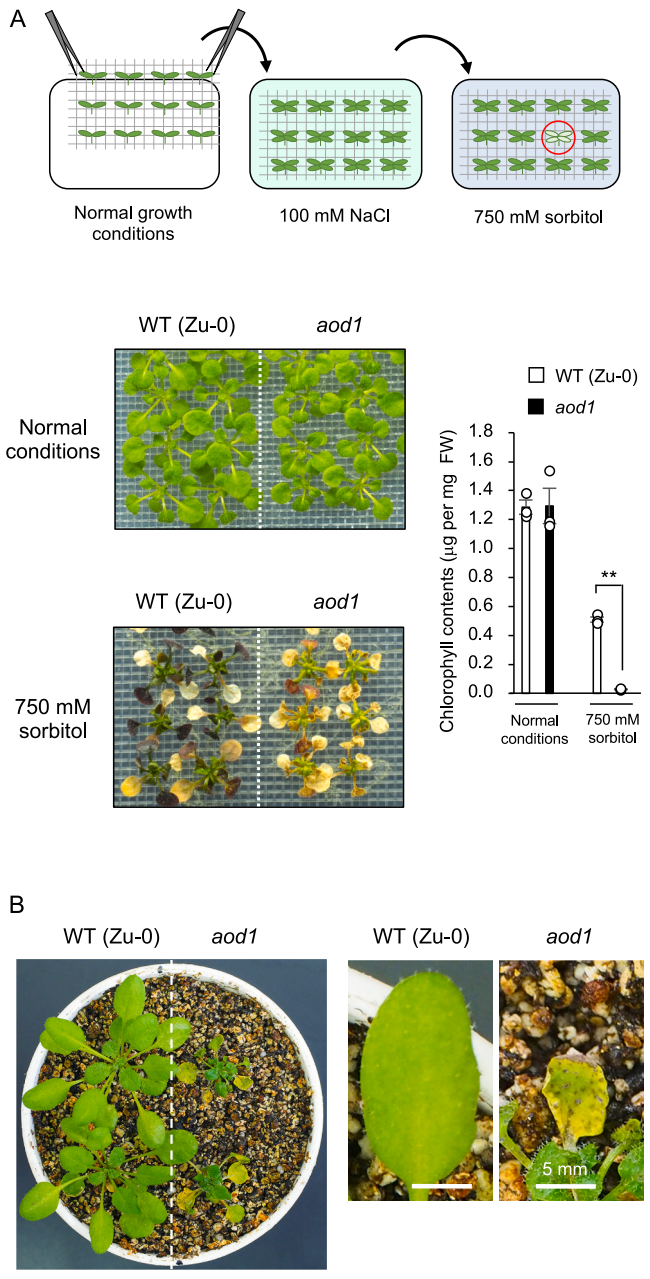

Fig. 1. Identification of the *acquired osmotolerance-defective 1* (*aod1*) mutant. (A) Flow chart of the acquired osmotolerance assay. Salt-acclimatized 2-week-old seedlings of Zu-0 were mesh-transferred to Murashige and Skoog (MS) agar plates containing 750 mM sorbitol and grown for 26 d. Seedlings that showed hypersensitivity (red circle) were selected as *aod* mutants. Upper photos: 14-day-old wild-type (WT) and M_3 *aod1* seedlings grown under normal conditions. Lower photos: acquired osmotolerance of WT and *aod1* plants. Right panel: chlorophyll contents of the corresponding □ WT and ■ *aod1* photos. FW, fresh weight. Differences between WT and *aod1* were analyzed by Student's *t*-test (mean ± SE, $n = 3$, $^{***}P < 0.001$). (B) Examples of 3-week-old WT and *aod1* plants grown in soil under normal conditions. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

are powerful tools for elucidating the complex PCD pathways associated with the plant immune system [9]. An Arabidopsis mutant called *constitutively activated cell death 1* (*cad1*) has a severe hypersensitive response (HR)-like cell death phenotype in the absence of pathogens [10]. *CAD1* encodes a protein containing the MACPF (membrane attack complex and perforin) domain of the C6–C9 components of the mammalian complement system and perforin, a pore-forming protein involved in innate immunity in animals [10,11]. The signature motif of MACPF is found in proteins in all kingdoms, including plants [12,13]. Arabidopsis has four MACPF motif-containing proteins, one of which, NECROTIC SPOTTED LESIONS 1 (NSL1), is also involved in immune responses [14]. The rosette leaves of the *cad1* mutant have dark brown cell-death lesions and constitutively express *PATHOGENESIS-RELATED 1* (*PR1*) and *PR2* with high accumulation of salicylic acid (SA), suggestive of a constitutively activated HR-like cell death phenotype [10]. Several alleles of *cad1* have been isolated; the homozygous *cad1-1* mutant is unable to produce seeds, whereas *cad1-5*, which carries a mutation causing a cysteine-to-tyrosine substitution at position 43 of CAD1, has a less severe phenotype than *cad1-1*, *cad1-2*, and *cad1-3* [10,15]. Bacterial NahG, an enzyme that degrades SA, suppresses cell death in *cad1-1*, whereas the introgression of a loss-of-function mutant of *NPR1* (*npr1*), a key transducer of SA-mediated plant immunity, into *cad1-1* partially restores cell death [10]. On the other hand, most of the enhanced immune phenotype observed in *cad1-5* is dependent on signaling through EDS1, as the small size and necrosis observed in *cad1-5* plants and the constitutive expression of *PR1* are suppressed in *cad1-5 eds1-2* [15]. EDS1 forms distinct signal-competent heteromeric complexes with SENESCENCE-ASSOCIATED GENE (SAG101) and PAD4 [16,17]. Interestingly, a mutation in *PAD4* failed to suppress most *cad1*-related immune phenotypes [18]. In contrast, the immune response induced by ACQOS under osmotic stress is suppressed by both EDS1 and PAD4, but not by transgenic expression of *NahG* or *npr1* [3]. Mutations in *CAD1* and *ACQOS* enhance the immune response through similar pathways; however, it is unclear whether *CAD1* is involved in osmotic stress or other nonbiological stress responses.

Here, we used a forward genetics approach to identify *aod* mutants by screening ion-beam-mutagenized seedlings of the osmotolerant Arabidopsis ecotype Zu-0. We isolated a novel mutant allele of *CAD1* as responsible for the *aod1* phenotype.

2. Materials and methods

2.1. Plant materials and growth conditions

Arabidopsis thaliana seeds (Zu-0, Col-0, or Bu-5) were sown on agar (0.8 % w/v) plates containing full-strength Murashige and Skoog (MS) salts with a vitamin mixture (10 mg L^{-1} myoinositol, 200 μg L^{-1} glycine, 50 μg L^{-1} nicotinic acid, 50 μg L^{-1} pyridoxine hydrochloride, 10 μg L^{-1} thiamine hydrochloride, pH 5.7) and 1 % w/w sucrose. Plates were sealed with surgical tape; the seeds were stratified at 4 °C for 4–7 d and then transferred to a growth chamber (80 μmol photons m^{-2} s^{-1}; 16/8-h light/dark cycle; 22 °C) for germination and growth. Zu-0 seeds were irradiated in an 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 at doses ranging from 25 to 250 Gy and assessed plant development. Doses of ≥200 Gy inhibited secondary leaf development or germination. Therefore, we irradiated seeds at 150 Gy in a single layer within a plastic bag.

2.2. Stress treatment for acquired osmotolerance assay

Seven-day-old seedlings grown on nylon mesh (990 μm) on an MS agar plate were mesh-transferred to a plate supplemented with 100 mM NaCl to grow for 7 d (Fig. 1A). They were then mesh-transferred to a plate supplemented with 750 mM sorbitol to grow for a further 26 d.

2.3. Abiotic stress assays

We mesh-transferred 10-day-old seedlings grown on nylon mesh (990 μm) on an MS agar plate to a plate supplemented with 650 mM sorbitol for 28 d (osmotic-shock stress), 200 mM NaCl for 13 d (salt-shock stress), or 10 μM paraquat for 28 d (oxidative stress). We

determined the chlorophyll content as described [19]. To explore the transcriptional response to osmotic stress in *aod1*, we analyzed the expression patterns of four osmotic stress marker genes (*RESPONSIVE TO DESICCATION 29A* [*RD29A*], *COLDREGULATED 15A* [*COR15A*], and *RESPONSIVE TO ABA 18* [*RAB18*]).

2.4. RNA extraction and qRT-PCR

Total RNA extraction and qRT-PCR were performed as reported [20]. Reverse-transcription PCR (RT-PCR) was performed at 94 °C for 2 min, followed by 27 cycles of 94 °C for 20 s, 58 °C for 20 s, and 68 °C for 1 min *Actin2* was used as an internal standard for both qRT-PCR and RT-PCR analyses. The PCR primers are listed in Supplementary Table S1.

2.5. Genetic mapping of the causative gene of aod1

We crossed the *aod1* mutant with Bu-5, an ecotype that shows acquired osmotic-stress tolerance [2], and selfed the resulting F_1 progeny to generate an F_2 population. Genomic DNA was prepared from individual F_2 plants with the recessive phenotype for use as PCR templates. We used the simple sequence-length polymorphism markers described in Ref. [2] for mapping. PCR conditions were initial denaturation at 94 °C for 2 min; 34 cycles at 94 °C for 20 s, 52–55 °C for 20 s, and 72 °C for 20 s; and final extension at 72 °C for 2 min. The microsatellites were fractionated in 5 %–7 % agarose gels, and the recombination frequencies (%) were calculated from the band pattern.

2.6. DNA library construction and sequencing of aod1

We performed DNA library construction and sequencing as described [8]. The read data were submitted to the DNA Data Bank of Japan (DDBJ) Read Archive (acc. No. PRJDB17926).

2.7. Detection of mutations in aod1

Mutations in the whole-genome sequencing data of both *aod1* and the Zu-0 WT were detected as described [8]. To confirm a 24-bp deletion at the transcript level, we performed RT-PCR by using primer sets 1 and 2, which amplified the deletion-rich exons 4 and 6, respectively.

2.8. Plasmid construction and transformation

For complementation analysis, we amplified the genomic region of *AOD1/CAD1* (2.0-kb upstream of the ATG initiation codon) by PCR with *Kpn*I and *Sma*I linker primers and cloned the region into the corresponding sites introduced into the binary vector pGHX-mGFP (pGreen 0029 background) with the 35 S promoter deleted. The construct was introduced into *Agrobacterium tumefaciens* strain GV3101, and plants were transformed by using the floral dip method. Primers for cloning are listed in Supplementary Table S1. Transgenic plants were selected on MS agar plates containing 200 μg mL^{-1} claforan and 20 μg mL^{-1} hygromycin. We transferred 10-day-old seedlings (T_1 plants) into soil pots.

2.9. Validation of mutated CAD1 as the gene responsible for the aod1 phenotype

To validate *CAD1* as the causal gene of *aod1*, we tried to generate transgenic lines by transforming *aod1* with *CAD1*, including its native promoter region, for a complementation test. However, we were unable to obtain a transgenic line because the *aod1* mutant has severely reduced fertility. Therefore, we transformed an F_2 line with a heterozygous *CAD1* mutation, which was generated by crossing *aod1* and Pog-0 (used in the mapping).

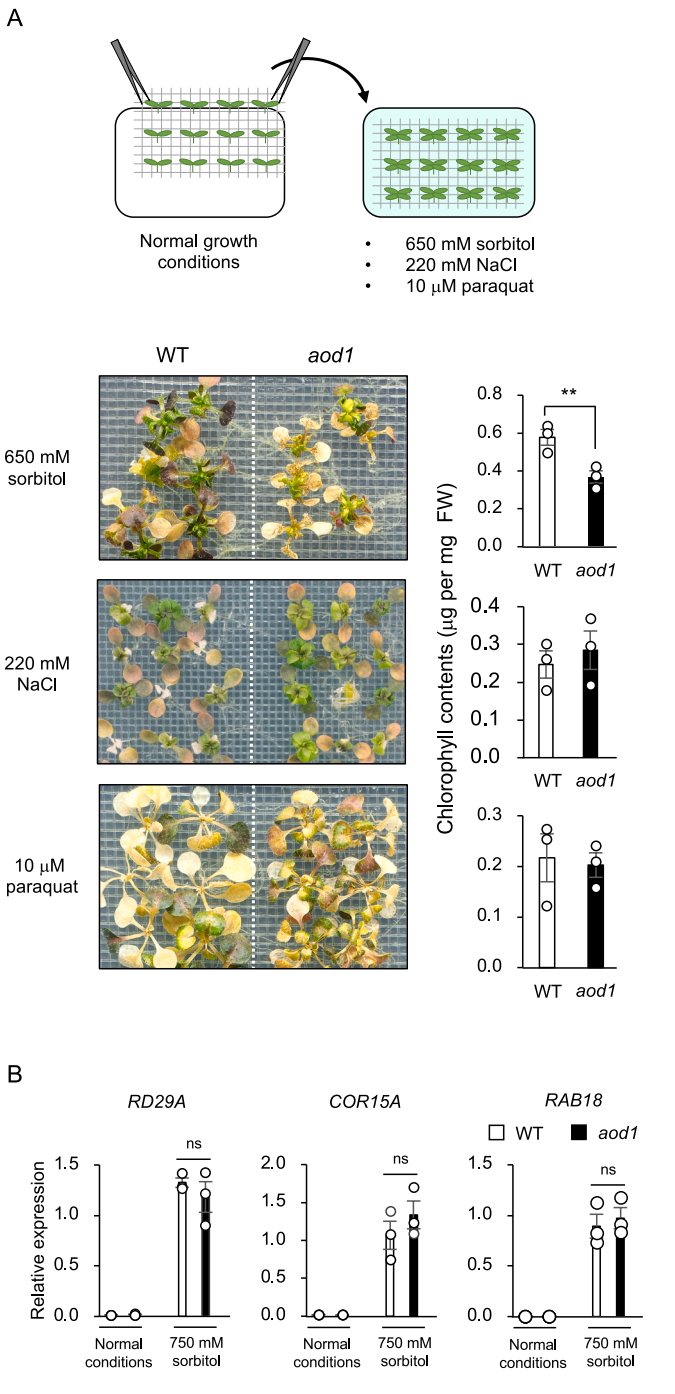

Fig. 2. Characterization of the *aod1* mutant.
(A) Top panel: Flow chart of the osmotic- and salt-shock and oxidative stress tolerance assays. Left: 10-day-old seedlings were mesh-transferred to MS agar plates containing 650 mM sorbitol and grown for 28 days (top), or 200 mM NaCl for 13 days (middle), or 10 μM paraquat (an inducer of oxidative stress) for 28 days (bottom). Right: chlorophyll contents of the seedlings shown at left. Differences between □ WT and ■ *aod1* were analyzed by Student's *t*-test (mean ± SE, $n = 3$, $^{**}P < 0.01$). (B) Expression profiles of the osmotic-shock-responsive marker genes in the WT and *aod1* seedlings under normal conditions (control) and under acquired osmotic stress conditions (100 mM NaCl for 7 days, followed by 750 mM sorbitol for 8 h); expression levels were determined by quantitative real-time PCR relative to those of *Actin2* (mean ± SE, $n = 3$). Differences between □ WT and ■ *aod1* were analyzed by Student's *t*-test (mean ± SE, $n = 3$).

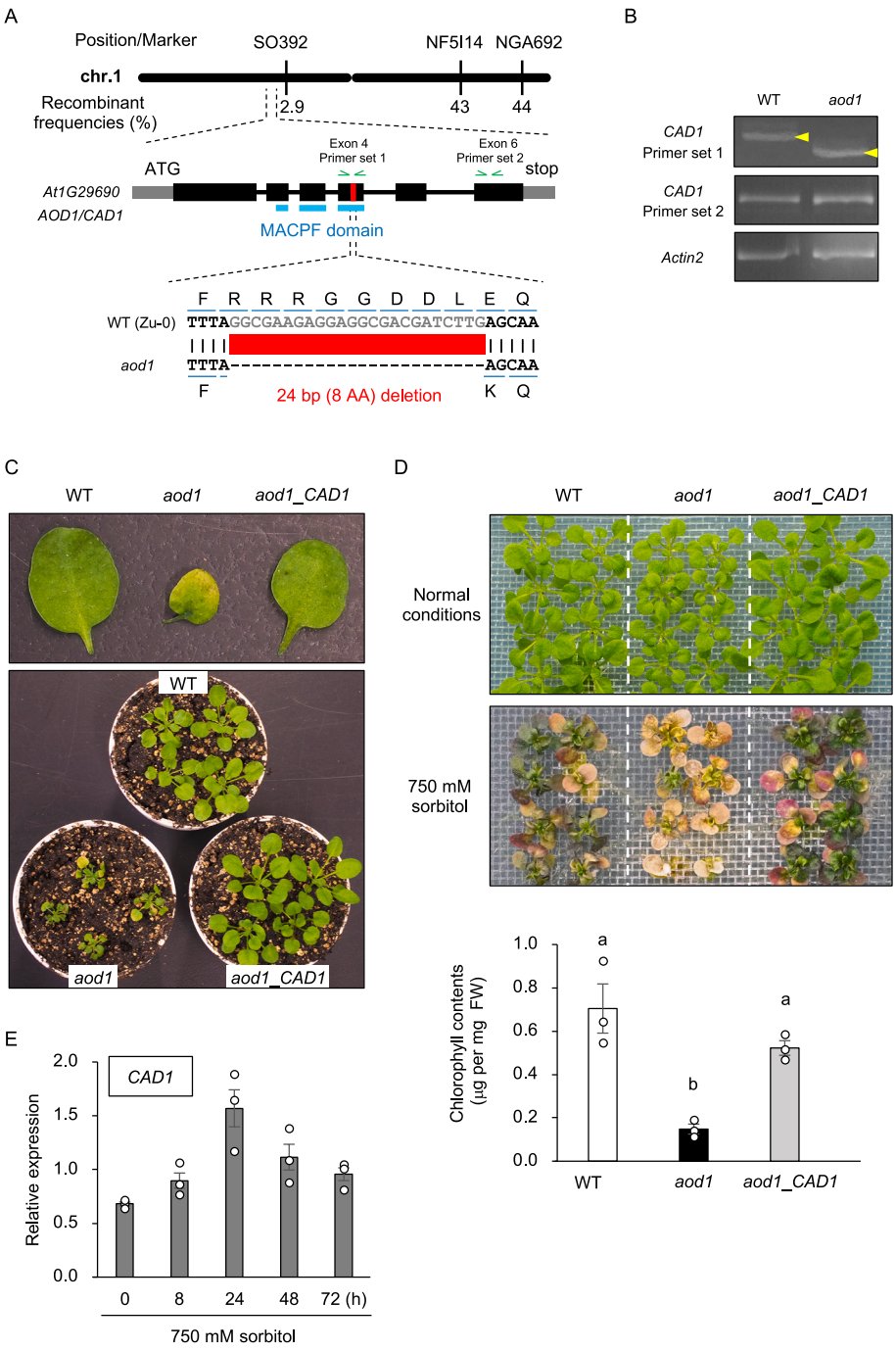

Fig. 3. Identification of the causal gene in *aod1*.
(A) High-resolution mapping of the causal locus in *aod1* by using F_2 progeny between *aod1* and Bu-5. *Arabidopsis* Genome Initiative (AGI) numbers are shown above the genes. The red bar shows the 24-bp (8 amino acid) deletion in *aod1*, resulting in a truncated protein. (B) RT-PCR-based genotyping of the 24-bp deletion in *At1g29690/CAD1* of the *aod1* genome. Primer set 1 with yellow arrowheads (in A) was designed to detect the 24-bp deletion in *aod1*, and primer set 2 was designed to detect DNA bands from both WT and *aod1* as a control. *Actin2* (*ACT2*) was used as the semiquantitative control. (C) Morphology of leaves and plants of 3-week-old WT, *aod1* and transgenic plants expressing *pCAD1::CAD1* in *aod1* mutant background (*aod1_CAD1*) plants grown in soil under normal conditions. (D) Upper photos: 14-day-old WT, *aod1*, and *aod1_CAD1* seedlings grown under normal conditions. Lower photos: acquired osmotolerance of the WT, *aod1*, and *aod1_CAD1* plants. Lower panel: chlorophyll contents of the corresponding □ WT, ■ *aod1*, and ■ *aod1_CAD1* plants under 750 mM sorbitol in the photos. FW, fresh weight. Bars labeled with different letters differ significantly ($P < 0.05$, one-way ANOVA with *post hoc* Tukey's HSD test, mean ± SE, $n = 3$). (E) Expression profiles of *CAD1* in WT under normal and osmotic stress conditions; expression levels were determined by quantitative real-time PCR relative to those of *Actin2* (mean ± SE, $n = 3$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.10. Immune response in aod1

The *cad1* mutant has a phenotype that mimics the lesions seen in HR with high expression of *PR* genes and high accumulation of SA [10]. To investigate whether the *CAD1* mutation in *aod1* leads to high expression of *PR* genes and PCD, we investigated the expression levels of *PR1*, *PR2* and *PR5* and detected cell death in plant leaves by trypan blue staining [21].

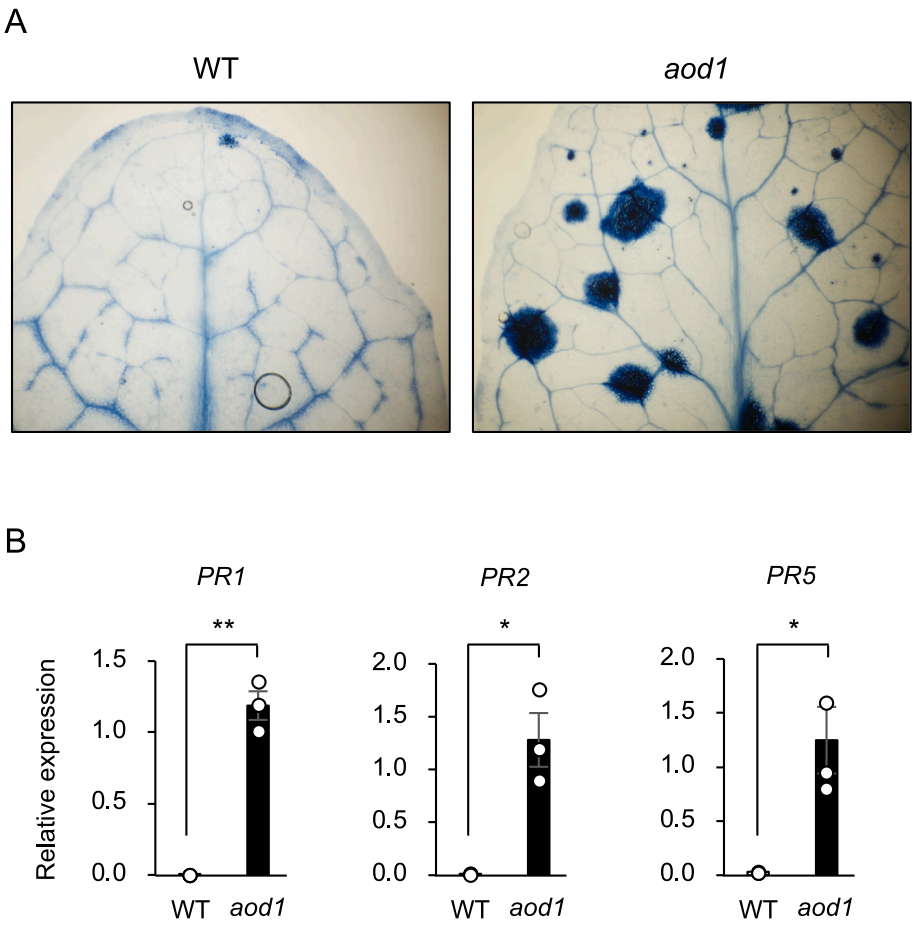

Fig. 4. Immune response in *aod1*.
(A) Trypan blue staining of leaves of WT or *aod1* seedlings under normal growth conditions. (B) Expression of pathogenesis-related genes (*PR1*, *PR2*, *PR5*) in Zu-0 WT and *aod1* plants under normal growth conditions; expression relative to *Actin2* was determined by quantitative real-time polymerase chain reaction. Differences between plants were analyzed by Student's *t*-test (mean ± SE, $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3. Results

3.1. Isolation and characterization of aod1 mutant

We screened ion-beam-mutagenized seed pools from the osmotolerant Zu-0 for mutants defective in acquired osmotolerance from the M_2 (second generation after mutagenesis) seeds. We screened 125 700 M_2 seeds derived from ~5800 M_1 seeds and isolated a mutant. To confirm the heredity of this mutant, we screened M_3 seeds obtained through self-pollination of the mutant. All M_3 seedlings exhibited defects in acquired osmotic tolerance (Fig. 1A). Under normal conditions, there was no significant difference in chlorophyll content between the WT and the mutant, but in the presence of 750 mM sorbitol, the chlorophyll content was significantly lower in the mutant than in the WT (Fig. 1A). We named the mutant *acquired osmotolerance defective 1* (*aod1*). The *aod1* plants were smaller than the WT under normal growth conditions on MS agar medium (Fig. 1A), and spotted necrotic lesions were observed on their rosette leaves under normal growth conditions on soil (Fig. 1B).

To characterize the *aod1* mutant, we investigated its response to various abiotic stresses. Compared with the Zu-0 WT, *aod1* was defective in osmotolerance after direct exposure to osmotic shock, but it was similar in tolerance to salt and oxidative stresses (Fig. 2A). Expression of the osmotic stress marker genes *RD29A*, *COR15A*, and *RAB18* was induced under osmotic stress in both *aod1* and the WT at comparable levels (Fig. 2B).

3.2. Identification of the gene responsible for the aod1 phenotype

By using F_2 progeny, we mapped the locus responsible for the osmosensitive phenotype of *aod1* near the simple-sequence-length polymorphism marker SO392, in the middle of chromosome 1 (Fig. 3A). Mutations were detected in 1257 genes, including 909 genes with amino acid changes, throughout the *aod1* genome. A 24-bp deletion resulting in an 8-amino acid deletion was found in *At1g29690/CAD1* (Fig. 3A) in the MACPF domain of complement components and perforin proteins. The 24-bp deletion in exon 4, but not in exon 6, of *CAD1* was detected at the transcriptional level in *aod1* also, suggesting that *aod1* produces mRNA 24 bp shorter than the WT (Fig. 3B).

Plants of line *aod1_CAD1*, homozygous for the 24-bp deletion in *CAD1* and carrying the WT *CAD1* gene, reversed the growth defects of *aod1*, indicating that mutated *CAD1* is the gene causing the growth-impairment phenotype observed in *aod1* (Fig. 3C). They also restored the acquired osmotolerance to that of the WT (Fig. 3D), indicating that mutated *CAD1* is the gene causing the osmosensitive phenotype of *aod1*.

In Zu-0 WT, the transcription level of *CAD1/AOD1* was increased by osmotic stress (Fig. 3E).

3.3. Immune response in aod1

Despite normal growth conditions, rosette leaves of *aod1* showed increased cell death relative to those of WT plants (Fig. 4A).

Furthermore, transcript levels of *PR1*, *PR2* and *PR5* were significantly higher in *aod1* than in the WT, even under normal growth conditions (Fig. 4B).

4. Discussion

We isolated a Zu-0-background osmosensitive mutant, *aod1*, the causal gene of which is identical to mutated *CAD1*. The CAD1 protein negatively controls the SA-mediated pathway of PCD in plant immunity [10,18]. We previously identified *ACQOS* as the gene responsible for acquired osmotolerance. Arabidopsis accessions with functional *ACQOS* alleles (e.g., Col-0) cannot acquire osmotolerance, whereas those with non-functional alleles (e.g., Zu-0 and Bu-5) can [3]. ACQOS contributes to bacterial resistance in the absence of osmotic stress but induces detrimental autoimmunity under osmotic stress via EDS1 and PAD4, thereby reducing osmotolerance [3]. ACQOS-mediated immune responses are suppressed by mutations in *EDS1*, *PAD4*, *RAR1*, and *SGT1*, but not by *NahG* or by mutation in *SID2* or *EDS5*, which encode SA biosynthesis enzymes [3]. On the other hand, the immune responses activated by the *cad1* mutation are suppressed not only by mutations in *EDS1* or *PAD4* but also by *NahG* [10,18,22], indicating that the detrimental immunity caused by the *cad1* mutation is due to a more SA-dependent pathway than the ACQOS-induced immune response. In addition, mutations in *CCX4*, encoding a Ca^{2+} transporter, and *MPK1*, encoding an MPK3/6 phosphatase, activate an immune response under osmotic stress and thus impair osmotic tolerance in Arabidopsis [7,8]. Conversely, mutations in the nuclear pore complex, including in *NUP85*, inhibit the translocation of ACQOS from the cytoplasm to the nucleus in response to osmotic stress, enhancing osmotic tolerance in Arabidopsis [23]. These results suggest that suppression of immune responses under osmotic stress plays an important role in osmotolerance of Arabidopsis; increased *CAD1* expression in response to osmotic stress suggests that CAD1 helps to suppress immunity in response to osmotic stress.

The *cad1* mutation in *aod1* occurs within the MACPF domain. The *cad1* alleles isolated so far enhance immune responses and cell death [15]. The 24-bp (8-amino acid) deletion in the MACPF domain in *aod1* is also likely important for the function of CAD1: the *cad1S205F* mutant, also with a mutation within the MACPF domain, has a phenotype similar to that of the min*7 fls2 efr cerk1* quadruple mutant, which is defective in both pattern-triggered immunity and the MIN7 vesicle transport pathway; thus, CAD1 may be one of the downstream converging components of the pattern-triggered immunity and the MIN7 vesicle transport pathway [24]. Orthologous animal proteins with MACPF domains, as pore-forming proteins involved in innate immunity in animals, are thought to be responsible for membrane attack on bacteria [11]. As the stress assay used in this study was performed under sterile conditions, CAD1 may have a different function from bacterial attack. CAD1 suppresses immune responses under normal or osmotic stress conditions, and its target appears to be the endogenous immune response system rather than exogenous factors such as bacteria.

5Disclosures conflicts of interest

No conflicts of interest declared.

CRediT authorship contribution statement

Yusuke Murakoshi: Conceptualization, Data curation, Formal analysis, Investigation. **Yasutaka Saso:** Data curation, Formal analysis, Investigation. **Minamo Matsumoto:** Data curation, Formal analysis. **Kazuha Yamanaka:** Data curation, Formal analysis. **Izumi Yotsui:** Writing – review & editing. **Yoichi Sakata:** Writing – review & editing. **Teruaki Taji:** Conceptualization, Funding acquisition, Project administration, Supervision, Validation, Writing – original draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2024.150049.

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