



## Changes in ALDH Family and Patterns of ACS Gene Expression in Strawberry (*Fragaria × ananassa* Duch.) in Response to Acetic Acid Application under Saline Conditions

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### ABSTRACT

The exogenous application of acetic acid has been reported to enhance plant tolerance to abiotic stresses. This study investigated the role of acetic acid in improving tolerance to NaCl and elucidated the underlying molecular mechanisms. Paros strawberry plants were foliar-treated with acetic acid (1 and 2 mM) and exposed to NaCl (0 and 40 mM). The expression patterns of selected ALDH (aldehyde dehydrogenase) and ACS (acetyl CoA synthase) candidates were analyzed. The results revealed that ALDH311 and ALDH7B4 were strongly induced by salt stress and by the application of acetic acid under saline conditions. The expression levels of these isoforms were significantly higher in plants treated with acetic acid under salt stress compared to untreated controls. Under non-stress conditions, ACS expression increased in response to 2 mM acetic acid but decreased with 1 mM treatment. In conclusion, ALDH genes, particularly ALDH311, were significantly upregulated in strawberry plants treated with acetic acid under salinity stress. ALDH7B4 also displayed increased expression under these conditions, albeit to a lesser extent than ALDH311. The activation of these ALDH genes appears to be associated with a defense mechanism that mitigates stress-induced damage.

**Abbreviation:** Acetyl CoA synthase (ACS), aldehyde dehydrogenase (ALDH), Pyruvate decarboxylase (PDC)

### Introduction

The application of acetic acid has been shown to increase plant tolerance to various abiotic stresses, including salinity, in multiple species such as Arabidopsis, maize, rice, cassava, and common bean (Kim et al., 2017; Hossain et al., 2019; Utsumi et al., 2019). Acetate biosynthesis plays a critical role in stress responses, mediated by enzymes like pyruvate decarboxylase (PDC) and aldehyde dehydrogenases (ALDHs) (Kim et al., 2017). ALDHs are widely expressed in plants

in response to a range of stresses, including salinity, drought, heat, waterlogging, oxidative stress, and heavy metal exposure (Tola et al., 2021). The upregulation of ALDHs is a common stress response (Singh et al., 2013), and their overexpression in transgenic plants has been shown to enhance stress tolerance, underscoring their importance in environmental adaptation (Deuschle et al., 2001; Kirch et al., 2004; Sunkar

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et al., 2003; Rodrigues et al., 2006; Stiti et al., 2011).

Aldehydes, often formed as intermediates in catabolic and biosynthetic pathways, can be toxic at high levels (Brocker et al., 2013). The ALDH superfamily, comprising NAD(P)<sup>+</sup>-dependent enzymes, catalyzes the irreversible oxidation of reactive aldehydes into their corresponding carboxylic acids (Kirch et al., 2004). Under optimal growth conditions, ALDHs participate in metabolic processes that regulate aldehyde levels and maintain cellular homeostasis (Vasiliou et al., 1999). These enzymes also support redox balance through their involvement in oxidoreductase activity and NADPH and NADH production (Kirch et al., 2004).

The Arabidopsis genome encodes 14 ALDH proteins spanning nine families, ranging from substrate-specific to multifunctional enzymes (Kirch et al., 2004). Transcriptomic analyses in Arabidopsis have shown that five ALDH isoforms—ALDH3H1, ALDH3I1, ALDH7B4, ALDH10A8, and ALDH10A9—respond to abscisic acid (ABA), salinity, and desiccation (Kirch et al., 2004; Missihoun et al., 2012). ALDH2B7, localized to mitochondria, enhances drought tolerance in plants (Rasheed et al., 2018), while ALDH3I1 mitigates aldehyde accumulation and oxidative stress (Zhao et al., 2017). Overexpression of ALDH3I1 in Arabidopsis has been associated with improved salinity tolerance (Sunkar et al., 2003). Coenzyme A (CoA) and acetyl coenzyme A (acetyl CoA) are pivotal in cellular energy metabolism, epigenetic regulation, signaling, and the metabolism of carbohydrates, lipids, and amino acids (Tubbs et al., 2015; Cai et al., 2011). Acetyl CoA synthetase (ACS), a plastidic enzyme, converts acetate and CoA into acetyl CoA, utilizing ATP as an energy source (Behal et al., 2002).

Strawberry is particularly sensitive to salinity compared to many other horticultural crops. Salt stress in strawberries induces severe effects, including nutrient imbalances, ion toxicity, reduced fruit quality and productivity, necrosis, and, in extreme cases, plant death (Jamali et al., 2016). While previous studies have demonstrated the positive effects of exogenous acetic acid on strawberry plants (Mirfattahi and Eshghi, 2020; Mirfattahi et al., 2023), the molecular mechanisms underlying these benefits under salt stress remain unclear. This study aimed to explore how foliar-applied acetic acid influences the expression of ALDHs and ACS in strawberry plants, with the goal of mitigating the detrimental effects of salt stress and improving plant resilience.

## Material and methods

### *Growing conditions and treatments*

Uniformly rooted daughter 'Paros' strawberry plants with similar crown diameter (*Fragaria × ananassa* 'Paros') were selected and sterilized using a 2% mancozeb fungicide. They were planted in 3 L pots filled with a cocopeat/perlite mixture (1:1 v/v) and grown under greenhouse conditions with a day/night temperature ranging from 23 ± 3/16 ± 3 °C and relative humidity (RH) maintained at 60 ± 5%. Light intensity was >800 mol m<sup>-2</sup> s<sup>-1</sup>.

To promote optimal vegetative growth and facilitate plant establishment, flowers were removed before the application of treatments. The plants were fertigated with ½ Hoagland nutrient solution every other day for a duration of two weeks, after which they were transitioned to the full Hoagland concentration (Hoagland and Arnon, 1950).

After full establishment of plants, when they had 5-6 fully grown leaves, plants were sprayed with 1 or 2 mM acetic acid solutions. At the same time salinity stress was initiated. Treatments included saline (40 mM NaCl) or non-saline conditions, i.e., control (Hoagland solution), 40 mM NaCl (S), foliar application with 1 mM acetic acid (AA), foliar application with 1mM acetic acid and exposure to 40 mM NaCl (AAS), foliar application with 2 mM acetic acid (Aa), and foliar application with 2 mM acetic acid and exposure to 40 mM NaCl (AaS).

After a period of thirty days, the leaves were harvested, promptly frozen in liquid nitrogen, and stored at -80 °C.

### *Identification of members of the strawberry ALDH family*

An exhaustive search of the GenBank non-redundant protein database was performed using the *Fragaria vesca* whole-genome v4.0.a1 assembly and annotation, available at [https://www.rosaceae.org/species/fragaria-vesca/genome\\_v4.0.a1](https://www.rosaceae.org/species/fragaria-vesca/genome_v4.0.a1). The queries included previously identified Arabidopsis ALDH sequences (Kirch et al., 2004), Pfam domain PF00171 (ALDH family), PS00687 (ALDH glutamic acid active site), PS00070 (ALDH cysteine active site), KOG2450 (aldehyde dehydrogenase), and KOG2451 (aldehyde dehydrogenase). The identified strawberry ALDH proteins were annotated using the guidelines of the ALDH Gene Nomenclature Committee (AGNC) (Zhang et al., 2012).

### *Phylogenetic analysis*

ClustalX in MEGA 7.0 enabled us to create a

multiple sequence alignment of ALDH protein sequences in strawberry (*Fragaria vesca*) and a number of other plant species using standard alignment sets. The resultant alignments were then used to generate the maximum likelihood algorithm within MEGA 7.0 software to generate phylogenetic trees. Subfamilies were subdivided using cluster analysis.

A total of 24 FVALDHs (ALDHs from *Fragaria vesca*) were identified. A comparison was made with the number of ALDHs reported in other plant species, including 16 ALDHs in *Arabidopsis thaliana* (Kirch et al., 2004), 23 in *Zea mays* (Jimenez-Lopez et al., 2010), 29 in *Solanum lycopersicum* (Jimenez-Lopez et al., 2016), 21 in *Physcomitrella patens*, and 9 in *Chlamydomonas reinhardtii* (Wood and Duff, 2009).

### **Validation of the selected genes in *Fragaria ananassa***

To identify homologous genes in *Fragaria × ananassa* and examine the candidate genes in *Fragaria vesca*, a de novo assembly was conducted using Trinity software on SRX3003891. Subsequently, the sequence similarity between these two species was confirmed through a local BLAST analysis (<https://ftp.ncbi.nlm.nih.gov/blast/db/>)

### **ALDH and ACS candidates for qRT-PCR**

Of the ten candidates of the ALDH gene family, three were evaluated. These genes were selected based on previous studies (Kirch et al., 2004; Kim et al., 2017). The primers (Table 1) were designed by Primer3 (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) and NCBI Primer-BLAST was used for checking their specificity.

**Table 1.** Primer for expression pattern analysis.

Gene Name	Primer for qRT-PCR	Product length (bp)	References
FaALDH2B7-F	GAGCGACACTGGAAACAGGA	20	This study
FaALDH2B7-R	GCACTGGGCCAAAGATCTCA	20	
FaALDH7B4-F	ATTCTGCAGTAGCCGCTCTC	20	This study
FaALDH7B4-R	TCAGTTCGGGCTTCGTTGAA	20	
FaALDH3I1-F	GTGGGTATTGGAAGCACCGA	20	This study
FaALDH3I1-R	AGAAGGAGGCCGAAGAGACT	20	
FaASC-F	AGGTCTCTAATGCGCGATGG	20	This study
FaASC-R	AATAGGCTCGCCTACGCTTC	20	
FaOLP2-F	GTTGACGGGTTCAATGTGGC	20	<a href="https://www.uniprot.org/uniprot/Q9SBT2">https://www.uniprot.org/uniprot/Q9SBT2</a>
FaOLP2-R	ATATCCGCCGTGCACTGAAT	20	
FaActin1-F	TTCACGAGACCACCTATAACTC	20	(Zhang et al., 2018)
FaActin1-R	GCTCATCCTATCAGCGATT	19	

### **Gene expression analysis**

For qRT-PCR validation analysis, total RNA was extracted from mature leaves using the Irizol RNA extraction kit (RNA Biotechnology Co., Iran). cDNA was synthesized using the RB MMLV reverse transcriptase kit (RNA Biotechnology Co., Iran). Three biological and three technical replications were considered for each sample per treatment. Quantitative real-time PCR was performed with the 7500 Real-Time PCR System (Applied Biosystems, New York, USA) using RB SYBR qPCR (RNA Biotechnology Co., Iran). Gene-

specific primers were used for amplification of first-strand cDNAs (Table 1). The transcripts of the strawberry gene ACTIN were used as a reference (Zhang et al., 2018). Linear regression of log (fluorescence) per cycle number was used for calculating RT-qPCR efficiency and was an indicative template concentration for each sample as described by Deng et al. (2014). Actin was used as an internal control. The relative expression level of ALDHs and ACS in strawberries was calculated using the 2<sup>(-Delta Delta C(T))</sup> method.

**Statistical analysis**

Data were analyzed using one-way ANOVA with SPSS software. Mean values of the measured parameters were compared using Duncan’s multiple range test ( $P \leq 0.05$ ). Pearson’s correlation coefficients were also calculated using SPSS software (IBM Corporation, Armonk, NY, USA) to determine relationships between variables.

**Results**

**Phylogenetic analysis**

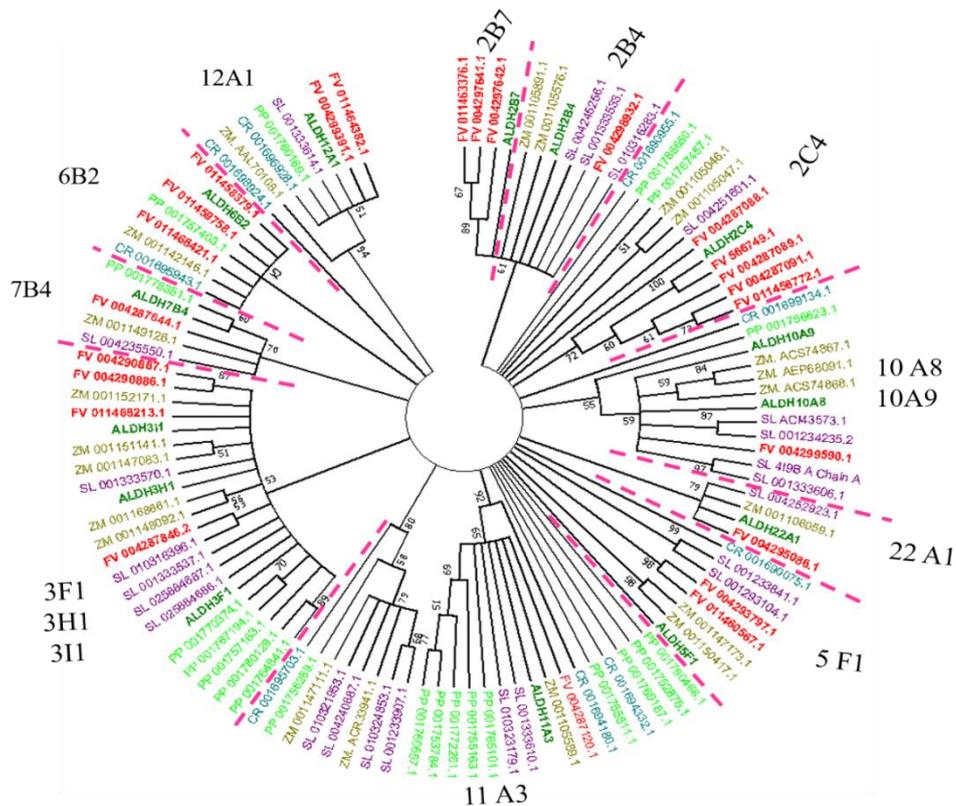
Phylogenetic relationships among strawberry ALDHs were examined, including a comparative analysis with the model plant *Arabidopsis thaliana*. A total of 97 full-length ALDH protein sequences were identified in *Fragaria vesca*. To construct a phylogenetic tree, known ALDH sequences were retrieved from *A. thaliana*, *Zea mays* (maize), *Solanum lycopersicum* (tomato), *Physcomitrella patens*, and *Chlamydomonas reinhardtii*.

Strawberry ALDH sequences were identified using BLAST analysis of known ALDH sequences. Putative strawberry ALDH sequences with E-

values under  $1e^{-6}$  were manually curated to confirm the presence of functional ALDH motifs, including Pfam00171, PS00687, and PS00070. Additionally, ALDH superfamily domains KOG2450, KOG2451, KOG2453, and KOG2456 were analyzed for further confirmation.

Figure 1 illustrates 23 groups combining ALDHs from the same families across different species. However, ALDH members belonging to the same family were not always grouped together. To gain insight into the evolutionary relationships among ALDH families, a phylogenetic tree was constructed using the neighbor-joining algorithm. This analysis utilized deduced protein sequences from several plant species, including three eudicots (*Arabidopsis*, strawberry, and tomato), one monocot (maize), and two lower plants (*Chlamydomonas reinhardtii* and *Physcomitrella patens*).

The results showed that most *F. vesca* ALDH families exhibit closer evolutionary relationships with *Arabidopsis* ALDH families than with ALDHs from other species.



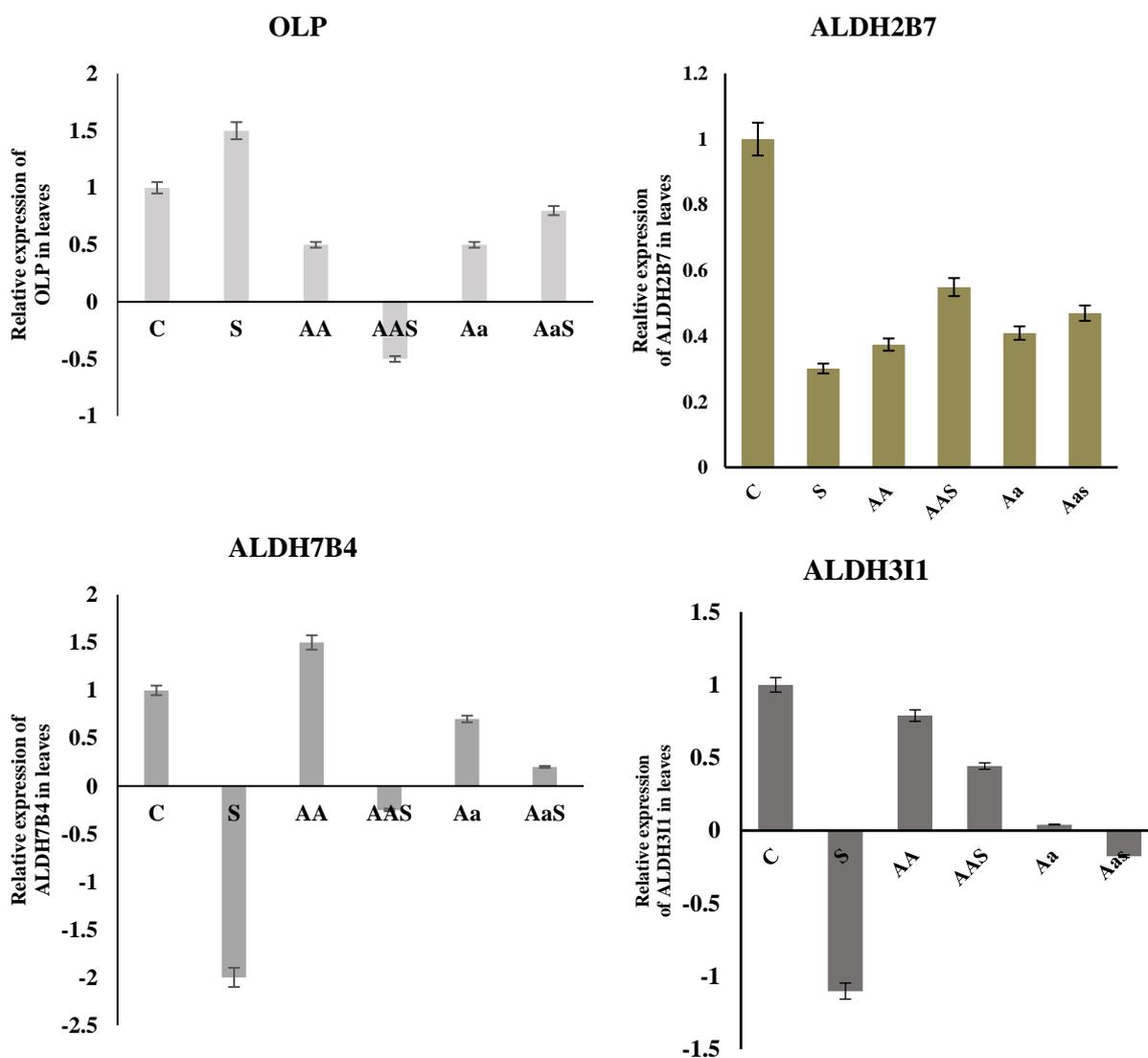
**Fig. 1.** Comparative phylogenetic analysis of strawberry ALDHs. Phylogenetic analysis was made using 97 ALDH proteins from *Solanum Lycopersicum* (SL), *Arabidopsis thaliana* (AT), *Zea mays* (ZM), *Physomitrella patens* (PP), *Chlamydomonas reinhardtii* (CR), and *Fragaria vesca* (FV).

### Analysis of salinity-induced expression of *ALDH2B7*, *ALDH4B7*, and *ALDH311*

The expression patterns of selected genes from the ALDH family displayed distinct variations in response to the treatments. As shown in Figure 2, the expression of *ALDH311* was downregulated following a 30-d NaCl treatment. In untreated plants sprayed with acetic acid, *ALDH311*

exhibited upregulation, exceeding the expression levels observed in the control group.

Under NaCl-induced stress conditions, *ALDH311* expression was significantly reduced, showing approximately a 77% decrease compared to the control plants. However, this reduction was less pronounced in plants subjected to the AAS (acetic acid spray) and AaS (acetic acid with stress) treatments.



**Fig. 2.** RT-qPCR confirmation of *ALDH2B7*, *ALDH7B4*, *ALDH311*, and *OLP* expression in strawberry plant leaves. Treatments included foliar application of 1 mM acetic acid under non-saline condition (AA), foliar application of 1 mM acetic acid under 40 mM NaCl (AAS), foliar application of 2 mM acetic acid under non-saline condition (Aa), foliar application of 2 mM acetic acid under 40 mM NaCl (AaS), 40 mM NaCl only as salinity stress (S), and Hoagland solution as the control (C). Error bars represent standard deviation (n = 3).

Distinct expression patterns were observed for the *OLP* gene, as shown in Figure 2. A notable decrease in *OLP* expression was observed in response to acetic acid treatment under both non-stress and stress conditions. Among the

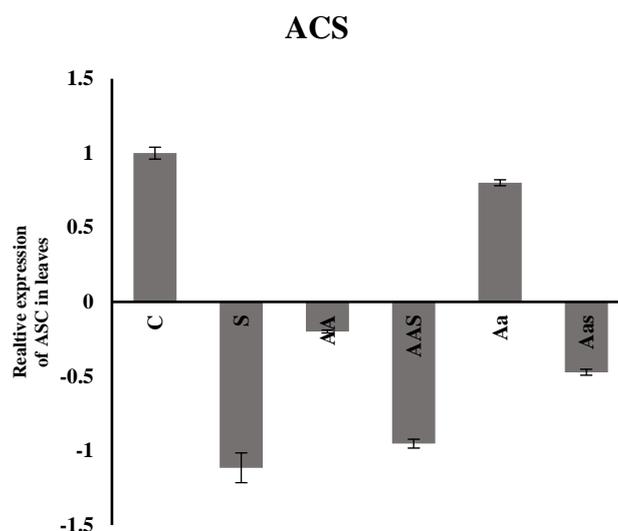
treatments, the highest expression level of *OLP* occurred in response to salinity stress (S treatment), while the lowest expression level was recorded in the AAS treatment. In contrast, the expression of *ALDH2B7* remained largely

unchanged under both stress and non-stress conditions, as indicated in Figure 2.

For ALDH4B7, an increase in expression was observed in non-stressed plants treated with acetic acid (AA and Aa treatments) compared to the control. However, under salinity stress (S treatment), the expression of ALDH4B7 decreased. This reduction was less pronounced in the AAS and AaS treatments compared to the S treatment alone (Fig. 2).

### ***Analysis of salinity-induced expression of ACS***

Under optimal growth conditions, the S and AAS treatments exhibited similar suppressive effects on ACS expression. Acetic acid generally enhanced the expression of ACS in plants treated with 2 mM acetic acid under non-stress conditions (AA), as shown in Figure 3. Conversely, spraying plants with 1 mM acetic acid (Aa) resulted in reduced ACS expression under non-stress conditions.



**Fig. 3.** RT-qPCR confirmation of *ACS* expression in strawberry plant leaves. Treatments included: foliar application of 1 mM acetic acid under non-saline condition (AA), foliar application of 1 mM acetic acid under 40 mM NaCl (AAS), foliar application of 2 mM acetic acid under non-saline condition (Aa), foliar application of 2 mM acetic acid under 40 mM NaCl (AaS), 40 mM NaCl only as salinity stress (S), and Hoagland solution as the control (C). Error bars represent standard deviation (n = 3).

## **Discussion**

Microarray and biochemical analyses have shown that acetic acid upregulates the *ALDH* gene family (Kim et al., 2017). Members of this family play a critical role in the biosynthesis of acetic acid in plants and are ubiquitous across yeasts, plants, and animals (Rasheed et al., 2018). The antioxidant activity and oxidative stress induced by environmental challenges increase the expression of specific *ALDH* genes, thereby enhancing cellular protection. While *ALDH* genes have been extensively characterized and analyzed in various plants, a genome-wide identification and comprehensive analysis of this gene family in strawberries has not been previously reported.

In the present study, 24 *ALDH* genes were identified in the strawberry genome and grouped into nine *ALDH* families (2, 3, 5, 6, 7, 10, 11, 12, and 22), following the criteria established by the *ALDH* Gene Nomenclature Committee (AGNC)

(Zhang et al., 2012). Although plant *ALDH*s can be classified into 14 families, only nine are represented in strawberries.

In *Arabidopsis*, the *ALDH2B7* gene, which participates in the acetic acid fermentation pathway, is upregulated in response to ABA application and abiotic stresses such as osmotic stress (Kim et al., 2017). Acetic acid treatment has also been shown to enhance drought tolerance in *Arabidopsis* (Kim et al., 2017). However, in this study, no significant increase in *ALDH2B7* expression was observed in strawberry plants subjected to salinity stress with acetic acid treatment. Conversely, the expression levels of *ALDH3I1* and *ALDH7B4* were transiently upregulated in response to acetic acid treatment. The molecular differences between strawberries and *Arabidopsis* could stem from two factors: (1) intrinsic variations in their responses to exogenous acetic acid; and (2) differences in experimental protocols, such as leaf spraying in

strawberries versus soil application in *Arabidopsis*. Previous studies have shown that ALDH3I1 and ALDH7B4 expression is strongly induced by dehydration, high salinity, and heat stress (Zhao et al., 2017). In the current study, ALDH7B4 exhibited lower expression levels than ALDH3I1 under both salt stress and acetic acid treatments, possibly requiring a longer duration of salinity stress than the one-month period used here.

These findings suggest that acetic acid treatment enhances salinity avoidance in strawberries by regulating the stress-responsive ALDH3I1 and ALDH7B4 genes. This is consistent with prior studies, which indicate that ALDH2B7 expression is more prominent under heat stress (Kim et al., 2017; Tola et al., 2021). In the ALDH family, ALDH7B4 and ALDH3I1 have been shown to play crucial roles in stress tolerance across both dicot and monocot species (Kirch et al., 2005).

In our study, the *Aa* treatment significantly enhanced *ACS* expression under non-stress conditions. Under salt stress conditions, acetic acid regulated *ACS* expression, resulting in increased gene expression compared to the *S* treatment. The rise in *ACS* expression under the *Aa* treatment may be attributed to the elevated acetate concentration.

Previous studies have demonstrated that the application of acetic acid can enhance drought stress tolerance in various plant species by modulating the jasmonic acid (JA) signaling pathway and histone acetylation (Kim et al., 2017). Notably, *ALDH2B7* and *PDC1* have been identified as critical components of both the JA signaling pathway and histone acetylation (Utsumi et al., 2019). While acetic acid has shown promise in improving drought stress tolerance, its effects and the underlying mechanisms in the context of saline conditions remain unexplored.

## Conclusions

The expression of *ALDH* genes, particularly *ALDH3I1*, was significantly induced in strawberry plants subjected to exogenously applied acetic acid and/or salinity stress. Additionally, an increased expression of *ALDH7B4* was observed following these treatments, though the induction of *ALDH7B4* and *ALDH2B7* was relatively lower. Notably, *ALDH3I1* exhibited a robust response to both acetic acid and salinity stress. These findings highlight the critical role of *ALDH* activation as a defense mechanism, mitigating the extent of stress-induced damage in strawberry plants.

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## Conflict of Interest

The authors indicate no conflict of interest in this work.

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