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Citation:

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Jasmonate biosynthesis regulates key events in barley vegetative and reproductive development

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Summary

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Received: 6 February 2026
Accepted: 4 August 2026

New Phytologist (2026)
doi: 10.1111/nph.71566

Key words: Allene Oxidase Cyclase, barley, development, gene editing, grain, jasmonate.

- Cereal grain is a global food staple. Filial tissues in grain, such as the embryo and endosperm, grow embedded in the maternal ovule and surrounding ovary wall. Maternal tissues provide nutrition and protection but are progressively reduced and degraded as filial tissues expand, driving grain expansion between floret hulls. While we know some enzymatic players, regulation of this process remains less understood. However, our previous work in barley, *Hordeum vulgare* spp. *vulgare* suggested a role for the phytohormone jasmonate (JA).
- We edited the barley *ALLENE OXIDASE CYCLASE* (*HvAOC*) gene encoding a JA biosynthetic enzyme. We examined *hvaoc* mutants for developmental phenotypes, genetic interactions and effects on the transcriptome.
- Defective *HvAOC* alleles reduced JA levels, and caused enhanced vegetative growth, faster flowering and lengthened grain, all phenotypes rescued by MeJA treatment. *HvAOC* control of grain length appears parentally-derived, consistent with enriched *HvAOC* expression in maternal compared to filial tissues. Grain dimension changes were uncoupled from hull size and correlated with larger endosperm cavities, more persistent maternal tissues and higher expression of genes linked to sucrose transport.
- This work provides the first genetic dissection of JA in barley and highlights roles in coordinating maternal tissues within grain.

Introduction

Grain from cultivated cereals provides more calories to the human diet than any other source. A complex multigenerational unit, grain originates from double fertilization of the ovule's embryo sac which generates the triploid endosperm and diploid embryo. These filial tissues grow embedded within the ovule's nucellus and further enclosed by inner and outer integuments, which collectively make up the seed. In cereals, the seed is surrounded by the ovary wall or pericarp, forming the 'caryopsis' or grain fruit. While the embryo grows and differentiates, endosperm nuclei divide into a coenocyte which cellularizes and fills with starch, driving caryopsis expansion between the lemma and palea hull (Olsen, 2020). At the same time, maternal tissues form nutritive and/or protective structures which ultimately die. The outer integuments disintegrate and the inner integuments and nucellar epidermis compress into a seed coat while pericarp cells proliferate, expand and subsequently crush into a protective case

during grain filling (Kellogg, 2016; Brinton & Uauy, 2019). Nucellar tissues specialize into structures which facilitate nutrient transport into the filial tissues. For instance, wheat and barley, dominant temperate cereal crops, form the nucellar projection (NP) along the grain ventral axis which acts as a central conduit for assimilate and which later undergoes programmed cell death (PCD) close to the filial boundary (Thiel *et al.*, 2008; Hands *et al.*, 2012; Domínguez & Cejudo, 2014). Collectively, these events shift the balance of tissues from the parental to the filial generation so that mature grain consists mainly of filial tissues held within reduced maternal tissues. Many alleles linked to changes in grain dimensions and/or weight also regulate ovary, nucellus, integument, pericarp and/or hull development (Yin & Xue, 2012; Ren *et al.*, 2018; Zhao *et al.*, 2018; Brinton & Uauy, 2019; Hao *et al.*, 2021; Jia *et al.*, 2021; Shoesmith *et al.*, 2021; Yang *et al.*, 2023; Long *et al.*, 2024; Wang *et al.*, 2024). However, the mechanistic links between genes regulating parental tissues and mature grain parameters are poorly

understood. For instance, agronomic alleles in rice reported to control grain size are only described to have a role in cell proliferation and/or expansion of hull organs (Riemann *et al.*, 2013; Ren *et al.*, 2018; Wang *et al.*, 2024).

We recently demonstrated that the miRNA-regulated transcription factor HvAPETALA2 (HvAP2) controls specific events in parental differentiation and degradation in the grain of the widely cultivated temperate cereal barley (*Hordeum vulgare* ssp. *vulgare*; Shoesmith *et al.*, 2021). First characterized as a repressor of internode growth and developmental progression in the spike (Houston *et al.*, 2013) and stem (Patil *et al.*, 2019), HvAP2 also restricts grain and lemma length, limits integument layer number and accelerates maternal tissue degradation, especially of the NP, an activity linked to the expression of the MADS-Box gene *HvMADS29*, a key regulator of NP differentiation in barley and rice (Yin & Xue, 2012; Shoesmith *et al.*, 2021). HvAP2-limitation of vegetative and grain development correlated with elevated jasmonate (JA)-responsive gene expression and sensitivity (Patil *et al.*, 2019; Shoesmith *et al.*, 2021), consistent with JA-related growth repression reported in many plants (Huang *et al.*, 2017).

JA controls other developmental processes, such as germination rate and flowering time, and plays a key signalling role in responding to biotic invaders and abiotic stresses (Wasternack & Hause, 2013; Campos *et al.*, 2014; Machado *et al.*, 2016; Huang *et al.*, 2017; Li *et al.*, 2025). Many insights about JA come from studying mutants defective in JA biosynthesis or signalling pathways which appear broadly conserved across land plants (Li *et al.*, 2021). JA and its metabolites, collectively called jasmonates, are oxylipin phytohormones originating from plastid-derived linolenic acid which is first oxygenated by 13-lipoxygenase (13-LOX), then converted into an epoxide by a 13-allene oxide synthase (AOS), and further cyclized by an allene oxide cyclase (AOC) to cyclopentenone (*cis*)-12-oxophytodienoic acid (OPDA). Reduction by OPDA reductase (OPR) and three cycles of β -oxidation in peroxisomes produce (+)-7-iso-jasmonic acid (Wasternack *et al.*, 2013), with further modifications yielding Jasmonoyl-isoleucine (JA-Ile), a major bioactive JA, as well as other active, inactive, or partially active compounds (Li *et al.*, 2021; Zhu *et al.*, 2021). Upon perception of bioactive JA, the coronatine insensitive 1 (COI1) receptor targets jasmonate ZIM-domain (JAZ) proteins for degradation, releasing JAZ-mediated repression of selected transcription factors, especially the MYC2 transcriptional activator central to many JA responses (Wasternack & Kombrink, 2010; Wasternack & Strnad, 2018). Recent genetic dissection in Arabidopsis showed that JAZ6 promotes seed size while downstream activators in JA signalling repress integument cell proliferation and ovule size (Hu *et al.*, 2021). JA restriction of seed size may be conserved in monocots as both *coi* mutants and overexpression of *OsJAZ11* in rice increase grain width and weight (Lee *et al.*, 2015; Mehra *et al.*, 2022). However, our understanding of how JA controls reproductive and grain development remains fragmentary in cereals, especially in wheat and barley.

To address this, we gene edited the *AOC* gene in barley. Predicted loss of HvAOC function led to elimination of JA,

enhanced vegetative growth, accelerated flowering and lengthened grain. Grain dimension changes correlated with larger and more persistent maternal tissues involved in nutrient transfer as well as increased endosperm, but no change in hull organ size. These results reveal a key role for JA in post-fertilization maternal tissue development and suggest that modulating JA levels may impact grain size and weight.

Materials and Methods

Germplasm and growth conditions

Barley plants were grown in glasshouses at the James Hutton Institute, UK, under long day photoperiod conditions (16 h : 8 h, light : dark, 18°C : 14°C) in standard cereal compost mix: 1.2 m³ Peat, 100 l sand, 1.5 kg Osmocote Exact Start, 3.5 kg Osmocote Exact 3–4 month, 2.5 kg Lime, Ca and Mg (each), 0.5 kg Celcote, 100 l Perlite and 280 g Intercept. Plants used for final phenotyping, grain developmental analyses and RNA-seq were sown in February 2024. Grain from *hvaoc n4/+* and Golden Promise individuals were first imbibed at 4°C for 2 d and then sown in well-watered 45-cell trays in the glasshouse. Once the plants had developed one fully expanded leaf, DNA was extracted for genotyping. Selected GP and *hvaoc n4* plants were transplanted into 3.5 l cylindrical pots at 23 d after sowing. F3 plants used to assess the *Zeo* and *hvaoc* interaction were sown in August 2023. Segregating *hvaoc-n4/+* plants used to investigate maternal control of grain traits were grown in a glasshouse at Zhejiang University, China. Plants were sown in November 2025 and harvested in May 2026. Individual plants were grown in 1-l pots filled with a 2 : 1 mixture of peat-based substrate and vermiculite. Plants were maintained under natural light and ambient glasshouse conditions throughout the growth period. For the germination test, surface-sterilized seeds ($n = 60$ per genotype) were germinated on moist filter paper in a controlled environment cabinet in 16 h : 8 h, light : dark cycle, set to provide a day photon irradiance of 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$, 70% relative humidity, a day temperature of 18°C and a night temperature of 18°C. Similarly, plants used for coleoptile length measurements under normal conditions were imbibed and germinated on moistened filter paper in plates within the same controlled growth cabinets. Germplasm details provided in Supporting Information Table S1.

Guide design, transformation, selection and screening

Gene-edited lines were generated in the Golden Promise cultivar by the John Innes Crop Transformation team. Two guides (Table S2) aligning to the 5' end of the first exon of the *HvAOC* gene model (HORVU.MOREX.r3.6HG0619890) were cloned into the dual guide acceptor vector (L2\Hv\35–36) and transformed into Golden Promise as described in Lawrenson & Harwood (2018). Five T0 plants were screened and edits detected in one line (line 01–01). Other T0 lines did not show edits in this generation. All T0 plants were grown, seed harvested and replanted for T1 generation. T2 seed from eight T1 plants (01–01, 01–02, 02–01, 03–01, 04–01, 04–03, 11–01 and

12–01) was sown and screened for the presence of Cas9 and edits in *HvAOC*. Two T3 lines with predicted loss-of-function edits in *HvAOC* (n4 and n7), which were also Cas9-free, were grown, confirmed by genotyping and selected for fine phenotyping. The presence of Cas9 and edited *HvAOC* were detected using primers targeted at the CaMV 35S promoter and Cas9 protein region.

Genotyping

Genomic DNA (gDNA) was extracted from young leaf tissue using the DNeasy Plant Mini Kit (Qiagen). Transgenic plants were genotyped for mutations in *HvAOC* and for the presence of the bcoCas9 transgene by PCR. *HvAOC* target sites were amplified using OneTaq 2× Master Mix with GC Buffer (New England Biolabs, Hitchin, UK). Each 15 µl reaction contained 7.5 µl of 2× master mix, 1 µl of GC enhancer (NEB), 0.5 µl of each primer, 1 µl of gDNA template and 4.5 µl of nuclease-free water. PCR cycling conditions consisted of an initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 58°C for 30 s and 68°C for 30 s. A final extension was performed at 68°C for 5 min. PCR products were purified with ExoSAP-IT (Applied Biosystems, ThermoFisher Scientific, Waltham, MA, USA) before Sanger sequencing. Two primer pairs were designed to detect the presence of the *Cas9* gene. Each 10 µl PCR reaction contained 5 µl OneTaq 2× Master Mix with Standard Buffer (NEB), 0.2 µl of each primer, 1 µl gDNA and 2.6 µl nuclease-free water. The cycling conditions consisted of an initial denaturation at 94°C for 30 s, followed by 35 cycles of 94°C for 30 s, 56°C for 30 s and 68°C for 54 s, with a final extension at 68°C for 5 min. The *Zeol1.b* allele was screened using GoTaq® Flexi DNA Polymerase following the manufacturer's recommendations. Each reaction contained 3 µl of 5× Green Flexi Buffer, 1.125 µl of MgCl₂, 0.75 µl of dNTP mix, 0.75 µl of each primer, 0.18 µl of GoTaq G2 enzyme and 1 µl of gDNA template. PCR amplicons were digested with SfaNI (NEB) to identify the *Zeol1.b* allele. Primer sequences for all genotyping and *Cas9* transgene detection are listed in Table S3.

Phenotyping

Coleoptile length was measured at 5 d after sowing (DAS) and germination rate recorded at 8 DAS. The number of tillers was counted at 32 DAS and 42 DAS. The DAS for anthesis was recorded for the first three tillers. Anthesis (0 d after pollination, DAP) was defined as the stage when anthers turned yellow and released pollen upon gentle pressure with tweezers. Grains were sampled at 0, 6, 10, 12, 16 and 18 DAP, and grains from the central spike portion were photographed, weighed and measured for length and width using IMAGEJ. Plant height, spike length, grain number for single spike (six spikes measured per plant) and spike yield were measured at mature stage. We note that some *hvaoc* plants died before flowering; any plants that died within 5 d after anthesis were excluded from the final grain phenotyping, whereas plants that survived beyond 5 d after anthesis were included in the analysis. Grain setting rate was calculated as the ratio of grain number : hull number. Grain dimensions and

lemma length were determined with MARVIN-Universal (MAR-ViNTECH GmbH, Wittenburg, Germany) and digital calipers.

Hormone measurements

Grain was washed with distilled water and put at 4°C in the dark for 2 d. Grain was placed onto damp filter paper in petri plates wrapped in aluminium foil and put in a cabinet set to 18°C to germinate. After 6 d, coleoptiles and inner shoot tissues were dissected off, flash frozen in liquid nitrogen and stored at –80 for several days before freeze-drying. Two individuals were pooled into individual tubes; then two to four tubes were combined into individual biological replicates ($n = 3$ per genotype). Extraction and LC-MS for JA, JA-Ile, OPDA, abscisic acid (ABA) and salicylic acid (SA) was adapted from the protocol described in Forcat *et al.* (2008). Internal standards were 10 ng of ²H₆-ABA, 2 ng of ²H₅-JA and 13.8 ng ²H₄-SA. Compounds were detected on an AB SCIEX QTRAP 6500 mass spectrometer in multiple reaction monitoring mode, using the following transitions: ²H₅-JA 214 > 62, JA 209 > 59, ²H₄-SA 141 > 97, SA 137 > 93, ²H₆-ABA 269 > 159, ABA 263 > 153, JA-Ile 322 > 130, OPDA 291 > 165. In addition, identification of OPDA was confirmed by enhanced product ion (EPI) MS–MS in negative ion mode with collision energy –40 V and collision energy spread 20 V.

Exogenous MeJA treatment

Exogenous MeJA (J2500; Sigma-Aldrich) treatments were applied to both seedlings and flowering plants. A 10 mM MeJA stock solution was prepared with few drops of ethanol and diluted with water to the desired concentrations. Coleoptile measurements were done on seedlings grown in the glasshouse. For seedlings, MeJA or water (mock control) was sprayed from the stage of coleoptile emergence. Two concentrations of MeJA (0.1 and 0.25 mM) were applied every 3 d, for a total of four applications. Due to the small size of the seedlings, a portion of the spray solution contacted the soil surface. Coleoptile length was measured at 14 DAS. For flowering-stage plants, we followed previous protocols for wheat and rice, and our earlier experiments in barley (Liu *et al.*, 2012, 2017; Li *et al.*, 2018; Patil *et al.*, 2019), MeJA was applied at a concentration of 1 mM. Spraying was initiated at anthesis (0 DAP) and was repeated every 3 d until 12 DAP. During application, the pots were wrapped with plastic film to prevent spray solution from entering the soil. MeJA solution was evenly applied to the aerial parts of the plants, including leaves, stems and developing spikes. Grains were sampled at 6 DAP, 18 DAP and at maturity. All spray solutions contained 0.01% (v/v) Tween-20 as a surfactant and water solution also contain same volume ethanol.

Grain histology

Grains from Golden Promise and mutant lines were collected at 6, 10, 12, 18 DAP and fixed overnight in formaldehyde acetic acid (FAA), dehydrated in a series of 70, 80, 90 and 100% (v/v)

ethanol and embedded in paraffin wax using Leica TP1020 Tissue Processor. Samples were sectioned to 10 µm thickness on microtome (RM2125; Leica Biosystems, Deer Park, IL, USA). Sections were stained in 0.5% Toluidine blue (w/v) after dewaxing and imaged by digital slide scanner (ZEISS, Axioscan, Oberkochen, Germany). For pericarp cell measurements, the ventral middle pericarp of fixed grain was dissected away manually, stained with 0.5% Toluidine blue and imaged with light microscope (MZ FLIII; Leica Biosystems).

Phylogenetic analysis

The barley *HvAOC* gene (HORVU.MOREX.r3.6HG0619890) was used as a query against the Ensembl Plants database (<https://plants.ensembl.org/index.html>) with default parameters to retrieve its homologues from other crop species, including monocots and eudicots. Phylogenetic relationships were inferred from amino acid sequences using a neighbour-joining method with 1000 bootstrap replications. Conserved motifs among the proteins were explored using the MEME motif search tool (<http://meme-suite.org/tools/meme>) using default settings specifying a maximum of 10 motifs. The resulting motif architectures were aligned and visualized with the phylogenetic tree using TBtools-II (<https://github.com/CJ-Chen/TBtools-II>).

HvAOC expression

Transcriptional expression data of *HvAOC* in various organs were obtained from EoRNA (<https://ics.hutton.ac.uk/eorna/index.html>) and BAR-ePlant Barley V3 (<https://bar.utoronto.ca/>). Except for the expression data of anthers, which were derived from Golden Promise, all other data were obtained from Morex cultivar. The shoot was sampled when it reached 10 cm in length, leaves were collected 2 months after sowing, and roots were sampled 4 wk after sowing. Lemma, palea and lodicule were collected 6 wk after flowering, rachis was collected 5 wk after flowering, and anthers were collected when they reached a length of 1.3–1.4 cm. Grains were collected at five time points after flowering and divided into three parts including maternal tissue, endosperm and embryo.

TUNEL assay

Paraffin-fixed caryopses at 6 and 10 DAP were selected for TUNEL assay using the Dead End Fluorometric TUNEL Kit (G3250; Promega) according to the manufacturer's instructions. Briefly, the paraffin was removed by xylene treatment, and the sections were rehydrated with an ethanol series. After treatment with proteinase K in PBS, the sections were incubated at 37°C for 60 min in the presence of rTdT. The TUNEL-positive control was set with DNase treatment. The green fluorescence of fluorescein (TUNEL signal) and red fluorescence of propidium iodide were analysed at 480 nm/520 nm and 620 nm/640 nm on the excitation/emission spectrum under the Zeiss ZT0 confocal microscope. For each genotype, three independent plants were selected and sections from three individual grains per plant were used for TUNEL detection.

Genetic analyses

The T3 *hvaoc-n4* homozygous and Cas-9-free line was crossed with the BWNIL938 containing the *Zeo1.b* gain of function *HvAP2* allele (Houston *et al.*, 2013). The F2 population was genotyped for *HvAOC* and *HvAP2* alleles. F3 seed was collected from each F2 individual. Selected F3 families were taken forward for genotyping, phenotyping and grain analyses.

RNA-seq

Caryopses of Golden Promise and *hvaoc-n4* at 6 and 12 DAP were used for RNA-seq. RNA isolation was performed using RNA Isolation and Purification Kit (Qiagen), followed with RNA integrity confirmation by Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). Library construction, sequencing, data pre-processing and differential expression analysis were performed as in Liu *et al.* (2022) with differentially expressed genes (DEGs) identified using a log₂ fold-change cutoff of 0.5. Gene Ontology (GO) enrichment analysis of the DEGs was performed on g:Profiler using a custom barley GO annotation as described in Liu *et al.* (2022). The enriched GO terms were summarized using ReviGo before the visualization.

Spatial transcriptome

Barley grains (cv Golden Promise) at 7 DAP and 10 DAP were dissected from hull and snap frozen in a liquid nitrogen-cooled isopentane bath for 1 min, subsequently embedded in optimal cutting temperature (Tissue-Tek). The grains were sectioned on a cryostat (Leica Biosystems) to 12 µm thick at −20°C. The sections were carefully placed on a prechilled Tissue Optimization (TO) or Gene Expression (GE) slide from Visium Tissue Optimization Slide and Reagent Kit (1000193; 10x Genomics, Pleasanton, CA, USA) and Visium GE Slide and Reagent Kit (1000187; 10x Genomics), respectively. As described by Peirats-Llobet *et al.* (2023), the sections were stained, imaged and incubated in pre-permeabilization solution at 37°C for 30 min. Following the GE workflow, cDNA from capture areas was synthesized and libraries were generated as described in the 10x Genomics User Guide (VisiumSpatialGeneExpression_UserGuide_RevE). Libraries were sequenced on an Illumina NextSeq 550 platform at Novogene (Malvern, Australia). Raw reads, bright-field image alignments and the latest barley reference genome (https://plants.ensembl.org/Hordeum_vulgare/Info/Index) were prepared as inputs for Space Ranger pipeline (<https://www.10xgenomics.com/software>) to generate transcript-spot matrices. Loupe Browser (v.8.0, 10x Genomics) was used for GE visualization.

Micro computed tomography analysis

Micro computed tomography (µCT) was used to visualize and quantify the internal and external architecture of the mature WT and *hvaoc-n4* grains. Ripened, dried, barley grains were mounted into a plastic straw and secured by wrapping in polyurethane.

Grains were scanned using a SkyScan 1272 (Bruker, Billerica, MA, USA) μ -CT unit. Scans were reconstructed using NRecon (v.2.2.0.6; Bruker). Scanning and reconstruction parameters can be found in Table S4. Reconstructions were aligned using data viewer (v.1.5.6.2; Bruker). Scanning and reconstruction parameters were kept consistent across all grains to ensure densities were comparable. Dragonfly 3D world (v.2024.1; Comet Technologies, Flamatt, Switzerland) was used to assign regions of interest (ROIs) and 3D analysis. The embryo, endosperm and aleurone layer were isolated using a combination of the ROI painter tool, the define range tool and morphological operations. The compute components tool was used to perform 3D analysis of the ROIs. The ruler tool was used for 2D analysis of length and width of the grains. Internal length reflects distance on a central sagittal section from the top edge of the aleurone to the bottom of the embryo, while internal width represents the distance from the widest part of the transverse section from the edge of the aleurone on each side. To determine crease dimensions, a central slice, at the widest part of the grain, was measured. The crease width and depth were determined using the ruler tool, taking the measure from the highest points of the endosperm lobes. The crease area was determined by defining an ROI of the cavity using the define range tool and the ROI painter tools. A representative image was chosen as the middle slice of the grain closest to the mean ($n = 5$). Stills and videos of the grain reconstructions were produced using CTVox (v.3.3.0; Bruker). Pseudo-colour was applied to indicate differences in density of the grains. Statistical analysis was carried out using GraphPad Prism (v.10.1.0), data set normality was established using a Shapiro–Wilk test, to which all μ CT data sets were deemed normal. An unpaired parametric t -test was carried out to determine the statistical significance between the WT and *hvaoc-n4* mature grains.

Statistics

Unless otherwise stated, all statistical analyses were performed using IBM SPSS Statistics 26 (IBM Corp., Armonk, NY, USA). Depending on the experimental design, two-tailed Student's t -tests, one-way ANOVA and Tukey's HSD *post hoc* tests were applied as indicated in the figure legends. Data visualization was carried out using Origin 2021 and R, and final figure assembly was completed in Adobe Illustrator 2021. Measurements were obtained from at least three independent biological replicates, as specified in the corresponding figures and no sample was measured more than once.

Results

HvAOC suppresses vegetative growth and developmental rate in barley

To explore the roles of JA in barley, we focused on the barley *ALLENE OXIDASE CYCLASE* (*AOC*) gene. Phylogenetic analyses confirmed that AOC is encoded by a single gene copy in many Poaceae, including barley (Fig. S1a). Evaluation of conserved motifs across land plants (Fig. S1b) suggested that AOC

motif structure is largely conserved across grasses while haplotype analyses of *HvAOC* over diverse barley germplasm, including cultivated, landrace and wild barleys revealed 18 haplotypes (Fig. S2). Of these, five HPs included non-synonymous SNPs (HPs_1, 2, 8, 9 and 15) and consisted entirely of wild barleys, while cultivated HPs were represented in seven HPs (HPs 3, 7, 11, 13, 14 and 15) with three exclusive to cultivated germplasm (HP 11, 14 and 16; Fig. S2), which together suggest that *HvAOC* likely shows no functional variation across cultivated barley. Publicly available expression data mined from EoRNA (Milne *et al.*, 2021) confirmed that *HvAOC* is expressed widely across different tissues (Fig. S3). As *HvAOC* is a single-copy gene whose ortholog encodes a biosynthetic enzyme early in the JA metabolic pathway and given its probable functional conservation, we selected *HvAOC* for gene editing to learn more about the role(s) of JA in barley growth and development.

We edited *HvAOC* in the Golden Promise cultivar using the cloning strategy and transgenic protocol described in Lawrenson *et al.* (2015) and Lawrenson & Harwood (2018), and screened the T2 generation for gene edits and identify plants lacking the *Cas9* transgene. We selected two putative impaired function alleles for further characterization, *hvaoc-n4*, where an insertion and deletion are predicted to cause a frame-shift and early stop codon, and *hvaoc-n7*, where a 1-bp insertion similarly should cause a frame-shift and early stop codon (Fig. 1a). Both lines were *Cas9*-free and propagated to the T4 generation for phenotypic comparison with parent cultivar Golden Promise. We measured JA and JA-Ile levels in Golden Promise and *hvaoc-n4* young shoot tissues ($n = 3$ bioreplicates per genotype) following the protocol described in Forcat *et al.* (2008). Mutant tissues showed a complete loss of JA, and JA-Ile was below the limit of quantitation (Table 1). However, we confirmed presence of OPDA at a low level (Fig. S4 (EPI MS–MS traces)) and suggest this represents racemic mixtures from spontaneous cyclization as shown in maize plants with loss of AOC function (Hunter *et al.*, 2025). Levels of other hormones (ABA, SA) were not significantly altered in mutant tissues. Germination rate was equivalent across Golden Promise, *hvaoc-n4* and *hvaoc-n7* (Fig. S5); however, *hvaoc-n4* and *hvaoc-n7* seedlings had 33% and 27% increased coleoptile length (Fig. 1b,c), respectively, with seedlings showing clear increases in growth by 8 DAS (Fig. 1d) compared to Golden Promise. We also noted longer subcrown internodes in *hvaoc-n4* compared to Golden Promise (Fig. 1e). By 32 DAS, *hvaoc-n4* plants displayed a more prostrate growth habit compared to Golden Promise plants but no change in tiller number at either 32 DAS or 48 DAS (Fig. 1f,g). Compared to Golden Promise, *hvaoc-n4* and *hvaoc-n7* were 15% and 18.7% shorter at maturity, respectively (Fig. 1h,i), and *hvaoc-n4* flowered 9.1 d earlier based on pollen dehiscence (Fig. 1j). We detected no change in spike length, grain number and grain setting percentage per spike across the genotypes (Figs 1k, S6). Taken together, loss of function *hvaoc* alleles lengthened coleoptiles, accelerated early seedling growth and flowered earlier compared to Golden Promise, suggesting a role for HvAOC in repressing early vegetative organ growth and stem elongation in barley.

HvAOC represses caryopsis growth independently from the lemma

Grain on maturing *hvaoc-n4* and *hvaoc-n7* spikes appeared more prominent with lemmas splayed open compared to Golden

Promise (Fig. 1k). Measuring mature grain parameters revealed that compared to Golden Promise, *hvaoc-n4* and *hvaoc-n7* grain was 13.6% and 13.3% longer and 14.4% and 5.3% thicker, respectively (Fig. 2a–e), while lemma length remained unchanged across genotypes (Fig. 2f), such that *hvaoc* grain bulged out to

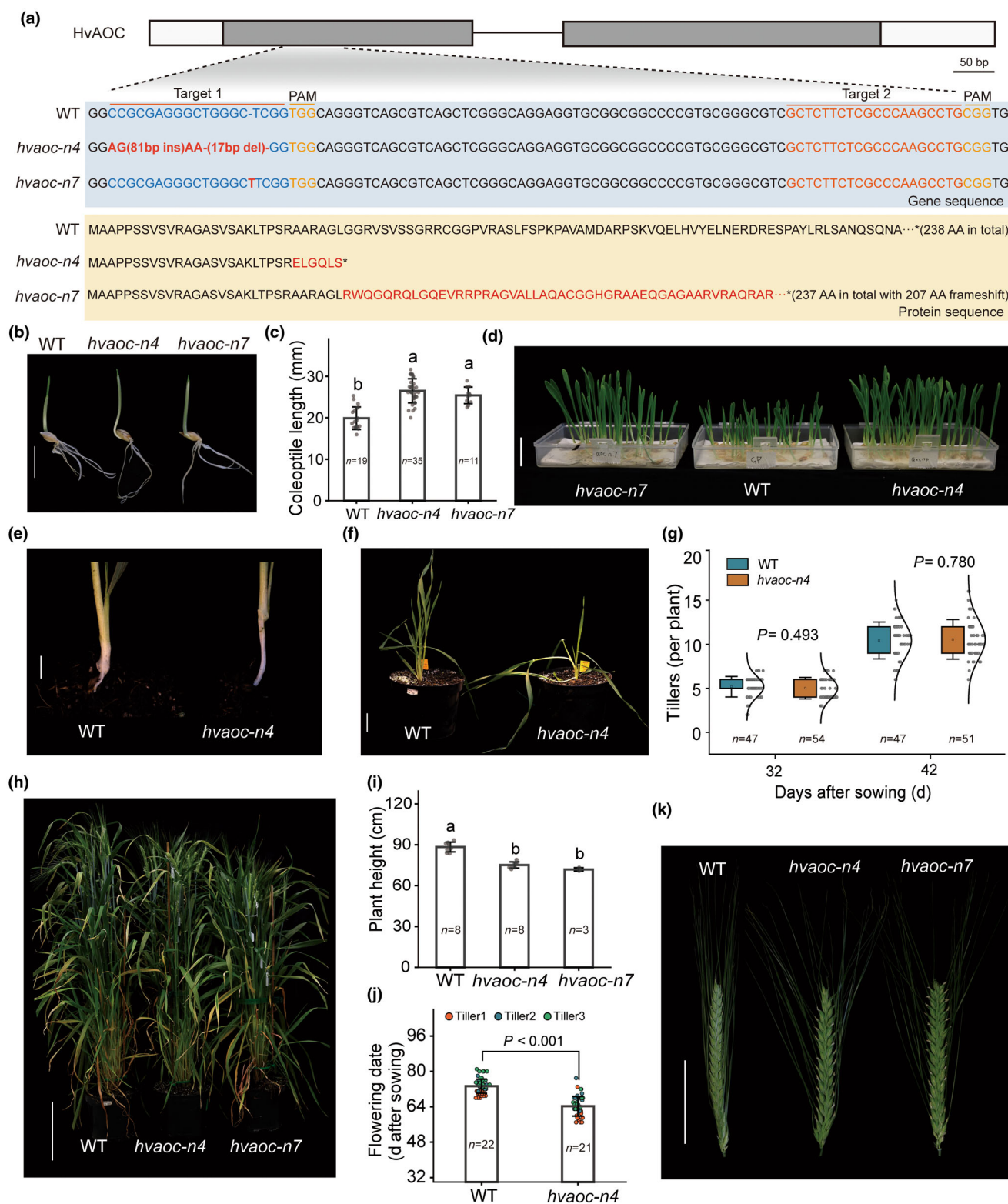


Fig. 1 Generation and general phenotypes of *hvaoc* mutants in barley. (a) *HvAOC* gene model with 5' region of exon1 magnified in the inset to show the Golden Promise (WT) sequences targeted by the guides (blue shaded box) and corresponding protein sequence (yellow shaded box). Edited regions and predicted changes in protein sequence are shown in red. (b) WT, *hvaoc-n4* and *hvaoc-n7* seedlings 5 d after sowing (DAS). Bar, 1.5 cm. (c) Coleoptile length of WT, *hvaoc-n4* and *hvaoc-n7* seedlings at 5 DAS. Grey dots represent individual biological replicates (*n* per genotype indicated under column). Bars represent SD. Different letters indicate significant differences ($P < 0.05$, one-way ANOVA, Tukey's HSD test). (d) WT, *hvaoc-n4* and *hvaoc-n7* seedlings at 8 DAS. Bar, 2 cm. (e) Subcrown internode of WT and *hvaoc-n4* at 17 DAS. Bar, 1 cm. (f) Growth habit of WT and *hvaoc-n4* plants at 32 DAS. Bar, 1 cm. (g) Tiller number of WT and *hvaoc-n4* plants at 32 and 48 DAS. Grey dots in represent individual biological replicates (Student's *t*-test with *P*-values as indicated per stage, *n* per genotype indicated on plot). Bars represent SD and the lower and upper box edges represent the first and third quartiles. (h) Fully grown plants of WT, *hvaoc-n4* and *hvaoc-n7* before significant senescence. Bar, 20 cm. (i) Plant height of WT, *hvaoc-n4* and *hvaoc-n7* mature plants. Grey dots represent individual biological replicates (*n* per genotype indicated on column). Bars represent SD. Different letters indicate significant differences ($P < 0.05$, one-way ANOVA, Tukey's HSD test). (j) Flowering time of the three tallest tillers in WT, *hvaoc-n4* and *hvaoc-n7*. Grey dots represent individual biological replicates ($P < 0.001$, Student's *t*-test, *n* per genotype indicated on plot). Bars represent SD. (k) Fully grown spikes of WT, *hvaoc-n4* and *hvaoc-n7* at 12 d after anthesis and before senescence. Bar, 5 cm.

Table 1 LC-MS Analyses of JA, SA and ABA levels in Golden Promise and *hvaoc-n4* seedling shoot tissues.

Sample ID	ABA	JA	JA-Ile	OPDA	SA
Aoc_B1	252	nd ^a	< 100 ^b	31 138	339
Aoc_B2	101	nd	< 50	22 408	164
Aoc_B3	93	nd	< 50	20 187	161
GP_B1	75	923	1759	150 477	167
GP_B2	67	586	1103	213 986	125
GP_B3	75	400	714	254 756	217
Units	ng g ⁻¹ DW	ng g ⁻¹ DW	Rel area g ⁻¹ DW	Rel area g ⁻¹ DW	ng g ⁻¹ DW

DW, dried weight.

^aNot detected.

^bBelow limit of quantitation.

prise open the lemma, generating a more open architecture. Accordingly, while Golden Promise grain length matched lemma length, *hvaoc-n4* and *hvaoc-n7* grain exceeded the end of the lemma by 1.6 and 1.2 mm, respectively. Uncoupling of lemma and grain length suggests that *HvAOC* may regulate grain dimensions independently from the lemma, in contrast to many other genes reported to control grain parameters. Grain weight was also altered, with 11.3% and 8.7% heavier grains in *hvaoc-n4* and *hvaoc-n7* compared to Golden Promise, respectively (Fig. 2g). Taken together, our data show that *HvAOC* is a major regulator of grain parameters, limiting grain length, depth and weight but not influencing lemma and palea elongation.

To explore the origin of grain differences, we tracked caryopsis development in *hvaoc-n4* and Golden Promise from 0 to 18 d after pollination (DAP; Fig. 2h–k). Compared to Golden Promise, *hvaoc-n4* caryopses were longer from 6 DAP onwards (Fig. 2i) while grain width and fresh weight increased compared to Golden Promise at earlier and later stages (Fig. 2j,k). Pericarp cell length and width in 12 DAP caryopses showed no difference between Golden Promise and *hvaoc-n4* suggesting that increased caryopsis length may represent increased cell number (Fig. 2l–n).

Phenotypes of *hvaoc* likely result from JA deficit

Since we theorize that *hvaoc* mutant phenotypes reflect a JA deficit, we predicted that adding JA may rescue these phenotypes. To test this, we treated both Golden Promise and the *hvaoc-n4* mutant with methyl jasmonate (MeJA) or mock solution and

then looked for evidence of phenotypic rescue. While Golden Promise and *hvaoc-n4* developed shorter coleoptiles in response to 0.1 and 0.25 mM MeJA, these lengths were equivalent between Golden Promise and *hvaoc-n4*, suggesting that MeJA completely rescued the *hvaoc-n4* long coleoptile phenotype (Fig. 3a,b). We also examined reproductive tissues treated with MeJA. Treatment of *hvaoc-n4* plants with 1 mM MeJA resulted in wild-type-looking spikes without 'prominent' grain exceeding lemma boundaries (Fig. 3c,d) and reduced grain length and thickness in both Golden Promise and *hvaoc-n4* (Fig. 3e–g). The length and thickness of MeJA-treated *hvaoc-n4* grain was equivalent to Golden Promise under mock treatment and removed genotype differences in grain width (Fig. 3f–h). Lemmas did not respond to MeJA treatment in either genotype (Fig. 3i). Treatment with 1 mM MeJA also reversed increased fresh caryopsis weight at 12 DAP and final grain weight in *hvaoc-n4* (Fig. 3j,k). Altogether, *hvaoc-n4* mutant vegetative and reproductive phenotypes resembled Golden Promise phenotypes following treatment with MeJA, indicating that coleoptile and grain phenotype were associated with JA levels and loss of *HvAOC* function may lead to phenotypes in *hvaoc-n4* due to a deficit in JA.

HvAOC regulates grain size parentally and independently of *HvAP2*

Our previous work showed that *HvAP2* restricts grain length which was associated with *HvAP2* promotion of JA-associated GE (Shoesmith *et al.*, 2021). To examine genetic interactions

between *HvAP2* and *HvAOC*, we crossed the BwNIL938 (Bowman near isogenic line 938) containing *Zeo1.b*, a *miR172*-resistant gain of function *HvAP2* allele (Houston *et al.*, 2013) with Cas9-free *hvaoc-n4* T3 plants. The Golden Promise cultivar background for the *hvaoc-n4* edits contains the mild *Zeo2* allele (Houston *et al.*, 2013). We genotyped F2 individuals for *Zeo* and

HvAOC alleles ($\chi^2 = 4.63$, $n = 64$, $P = 0.80$) and selected those fixed for either *Zeo1.b* or *Zeo2* allele and heterozygous at the *HvAOC* locus (i.e. segregating for Bowman *HvAOC* and the gene-edited *hvaoc-n4* alleles). We planted and genotyped F3 families from each selected F2. Each F3 family contains a mosaic Bowman and Golden Promise background. Within each family

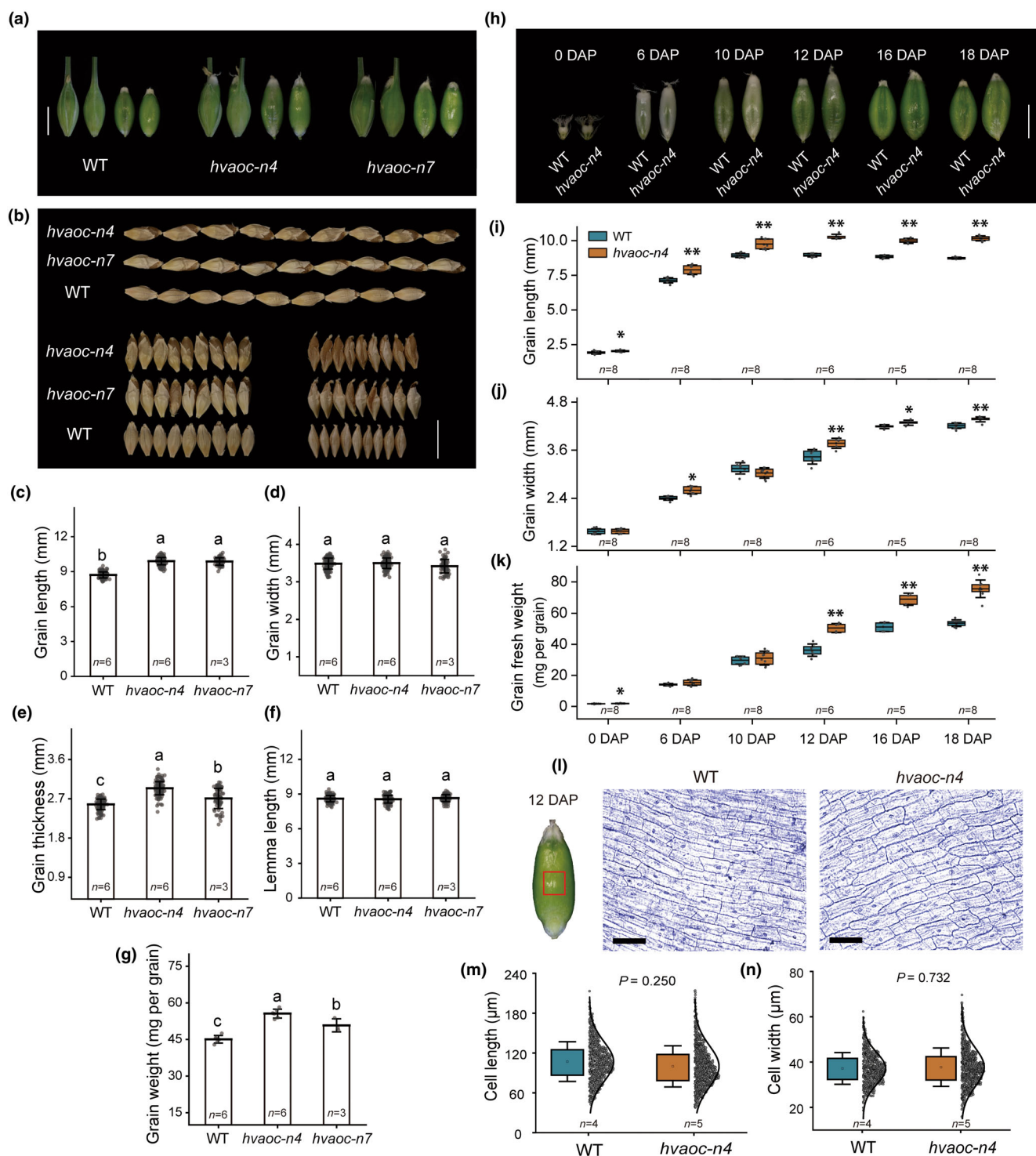


Fig. 2 *HvAOC* controls grain size traits and development in barley. (a) Caryopses removed from Golden Promise (WT), *hvaoc-n4* and *hvaoc-n7* spikes 12 d after pollination (DAP), pictured with and without the lemma. Bar, 0.5 cm. (b) Mature grain of WT, *hvaoc-n4* and *hvaoc-n7*. The two sets are arranged differently to highlight changes in different dimensions: the top set is aligned to compare grain width, whereas the bottom set is aligned to compare grain thickness. Bar, 1 cm. (c–g) Comparisons of mature grain length (c), grain width (d), grain thickness (e), lemma length (f) and grain weight (g) between WT, *hvaoc-n4* and *hvaoc-n7*. Grey dots in (c–f) represent single grain measurements (24 measurements per individual) from at least three biological replicates, while the dots in (g) represent individual biological replicates. Bars represent SD and different letters indicate significant differences ($P < 0.05$, one-way ANOVA, Tukey's HSD test, $n \geq 3$ individuals per genotype). (h) Caryopsis development in WT and *hvaoc-n4* from 0 d after pollination (DAP) to 18 DAP. Bar, 0.5 cm. (i–k) Comparisons of caryopsis length (i), width (j) and weight (k) between WT and *hvaoc-n4* from 0 DAP to 18 DAP. *, $P < 0.05$; **, $P < 0.01$ (Student's *t*-test, $n \geq 5$ individuals per genotype). The horizontal line represents the mean, the bars represent SD, and the lower and upper edges of the box represent the first and third quartiles, respectively. The dots represent individual biological replicates. (l) Comparison of cell size in the ventral pericarp of WT and *hvaoc-n4* at 12 DAP. Representative photo on left showing the ventral region analysed (red box). Centre and right panels show pericarp stained with Toluidine Blue. Bar, 100 μm . (m, n) Pericarp cell length and width. Grey dots indicate one cell size measurement (Student's *t*-test with *P*-values as indicated on the panels, $n \geq 4$ individuals per genotype, with three grain images per individual and ≥ 35 cell measurements per image).

(*Zeo2/Zeo2 hvaoc-n4/hvaoc-n4* F3, $\chi^2 = 0.40$, $n = 63$, $P = 0.82$; *Zeo1.b/Zeo1.b hvaoc-n4/hvaoc-n4* F3, $\chi^2 = 0.37$, $n = 60$, $P = 0.83$; Table S5), we identified and phenotyped *HvAOC/HvAOC*, *HvAOC/hvaoc-n4* and *hvaoc-n4/hvaoc-n4* siblings. We first compared *HvAOC/HvAOC* and *hvaoc-n4/hvaoc-n4* siblings fixed for either the *Zeo2* or *Zeo1.b* allele. *Zeo1.b* causes shorter internodes in the stem and spike (Houston *et al.*, 2013; Patil *et al.*, 2019). We detected no difference in spike density in the *Zeo1.b/Zeo1.b* siblings regardless of *HvAOC* or *hvaoc-n4* allele (Fig. 4a,c), suggesting that *HvAOC* does not influence *HvAP2* suppression of rachis (spike axis) internode length. However, *hvaoc-n4/hvaoc-n4* siblings combined with either *Zeo2* or *Zeo1.b* alleles showed slight increases in plant height (Fig. S7), suggesting that *hvaoc-n4* can partially compensate for *Zeo*-associated decreases in stem elongation. Grain formed on *Zeo2/Zeo2 hvaoc-n4/hvaoc-n4* and *Zeo1.b/Zeo1.b hvaoc-n4/hvaoc-n4* spikes was visible compared to other siblings (Fig. 4b), consistent with caryopses exceeding the lemma boundaries. Measuring grain length confirmed that *Zeo2/Zeo2 hvaoc-n4/hvaoc-n4* and *Zeo1.b/Zeo1.b hvaoc-n4/hvaoc-n4* developed longer grain than siblings with two copies of *HvAOC* (Fig. 4d). The type of *Zeo* allele made no difference to grain length in *HvAOC/HvAOC* individuals; however, grain from *Zeo1.b/Zeo1.b hvaoc-n4/hvaoc-n4* plants were shorter compared to *Zeo2/Zeo2 hvaoc-n4/hvaoc-n4* indicating that *Zeo1.b* can partially compensate for the grain-lengthening effect of *hvaoc-n4* (Fig. 4d). Genotypes showed no differences in grain width or thickness (Fig. 4e,f) or grain weight, except for *Zeo2/Zeo2 hvaoc-n4/hvaoc-n4* plants which yielded heavier grain (Fig. 4h). Interestingly, *Zeo2/Zeo2 hvaoc-n4/hvaoc-n4* florets had longer lemmas compared to other genotypes (Fig. 4g). Since *hvaoc-n4* did not change lemma length in the Golden Promise background, increased lemma length in *Zeo2/Zeo2 hvaoc-n4/hvaoc-n4* plants may reflect interactions between parental backgrounds or that *HvAOC* may influence lemma length in a *Zeo* allele-dependant manner. Taken together, gain of *HvAP2* function partially interfered with the *hvaoc-n4* ability to increase grain length and weight, suggesting that *HvAP2* restriction of grain length may depend on adequate activity of *HvAOC*, and therefore, the JA pathway.

We also examined grain parameters in F3 plants genotyped as *HvAOC/hvaoc-n4* and fixed for either *Zeo* allele; all grain from these plants will have maternal tissues with the *HvAOC/hvaoc-n4* genotype but contain embryos with these genotype distributions: 25% *hvaoc-n4/hvaoc-n4*, 50% *HvAOC/hvaoc-n4* and 25%

HvAOC/HvAOC. Grain from *HvAOC/hvaoc-n4* siblings with either *Zeo* allele did not show visible differences or different populations of grain length compared to grain from *HvAOC/HvAOC* siblings (Fig. 4i,j), while grain from *hvaoc-n4/hvaoc-n4* siblings showed longer grain as expected (Fig. 4d,j,k), suggesting changes in grain length due to *hvaoc-n4/hvaoc-n4* are parentally derived. We also examined grain from *HvAOC/hvaoc-n4* individuals in the uniform Golden Promise background compared with Golden Promise controls. We detected no differences between these genotypes, or any evidence for segregation in the grain from the *HvAOC/hvaoc-n4* plants (Fig. S8). Taken together, our data indicate that *HvAOC* function in parental tissues regulates grain dimensions.

HvAOC controls maternal tissue differentiation and degradation

Differences in grain parameters between Golden Promise and *hvaoc-n4* alleles emerged early in caryopses growth (Fig. 2). To understand more about the relationship between *HvAOC* function and grain development, we examined *HvAOC* expression in the BAR-ePlant Barley V3 RNA-seq dataset from developing caryopses (<https://bar.utoronto.ca/>; Kovacic *et al.*, 2024), which revealed higher expression of *HvAOC* in maternal vs filial grain tissues (Fig. 5a). We next examined *HvAOC* expression in caryopses using a Golden Promise spatial transcriptome at 10 DAP (Fig. 5b). *HvAOC* transcripts were detected across the grain, and noticeably enriched in vascular bundles, regions flanking the vascular bundle and the NP (Fig. 5b), tissues with important roles in parental-to-filial nutrient transfer (Radchuk *et al.*, 2023).

Semi-thin sectioning of developing caryopses in Golden Promise compared *hvaoc-n4* mutant revealed profound anatomical differences. Grain development in cereals is marked by the appearance of a so-called endosperm cavity, associated with PCD of maternal tissues, especially the NP (Radchuk *et al.*, 2006; Domínguez & Cejudo, 2014; Shoesmith *et al.*, 2021). The cavity expanded progressively from 6 to 18 DAP in both genotypes (Fig. 5c); however, cavity area of 12 and 18 DAP caryopses was 3.5- and 3.9-fold larger in *hvaoc-n4* caryopses than Golden Promise, respectively (Fig. 5c,e). By 18 DAP, endosperm area was 21.6% greater in *hvaoc-n4* caryopses than Golden Promise (Fig. 5c,f). We did not observe any significant differences in NP

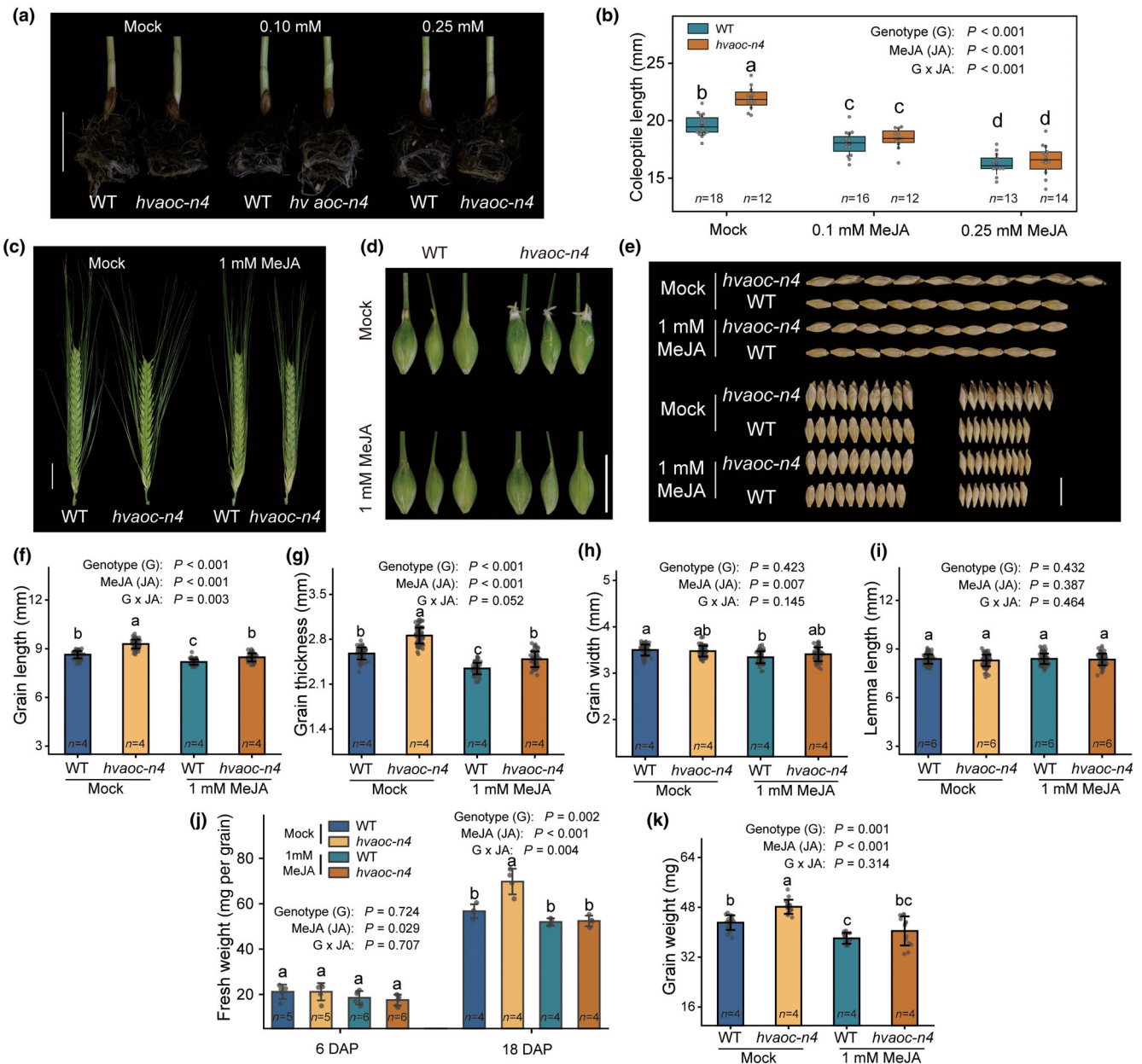


Fig. 3 MeJA treatment on coleoptile and grain parameters in WT and *hvaoc-n4* barley plants. (a, b) Representative images of coleoptile length in WT and *hvaoc-n4* after mock and MeJA treatment. Bar, 2 cm. (b) Quantification of coleoptile length in WT and *hvaoc-n4* after mock and MeJA treatment. Grey dots represent biological replicates. Different letters indicate significant differences ($P < 0.05$, Two-way ANOVA, Tukey's HSD test, $n \geq 4$). The horizontal line represents the median, the hollow squares represent the mean, the bars represent SD, and the lower and upper edges of the box represent the first and third quartiles, respectively. The dots represent individual biological replicates. (c, d) Spike and grain morphology of WT and *hvaoc-n4* at 12 d after pollination (DAP) following mock or MeJA treatment. Bar, 2 cm. (e) Representative images of mature grains from WT and *hvaoc-n4* treated with mock or MeJA. Bar, 1 cm. (f–k) Comparison of mock and MeJA treatments in WT and *hvaoc-n4* for grain length (f), grain thickness (g), grain width (h), lemma length (i), caryopsis fresh weight at 6 DAP and 18 DAP (j) and grain weight (k). Grey dots in (g–i) and (k) represent biological replicates, while grey dots in (j) represent one grain measurement taken from $n \geq 4$ biological replicates. Different letters indicate significant differences ($P < 0.05$, Two-way ANOVA, Tukey's HSD test, $n \geq 4$).

area between genotypes in 10, 12 or 18 DAP caryopses (Fig. 5g). By contrast, the vascular bundle (VB) area increased 55.3% in *hvaoc-n4* 12 DAP caryopses compared to equivalently staged Golden Promise (Fig. 5h). Notably, the region flanking the vascular bundle (FVB) at 10, 12 and 18 DAP increased in area by 16.9%, 48.6% and 17.7% in *hvaoc-n4* caryopses compared to

Golden Promise, respectively (Fig. 5d,i). Altogether, loss of HvAOC enhanced the area and integrity of the VB and FVBs as well as cavity area, putatively increasing assimilate transfer into the filial tissues. Since the endosperm cavity appears concomitant with maternal tissue PCD, we asked whether *HvAOC* regulates PCD in developing grain. We used TUNEL assays which label

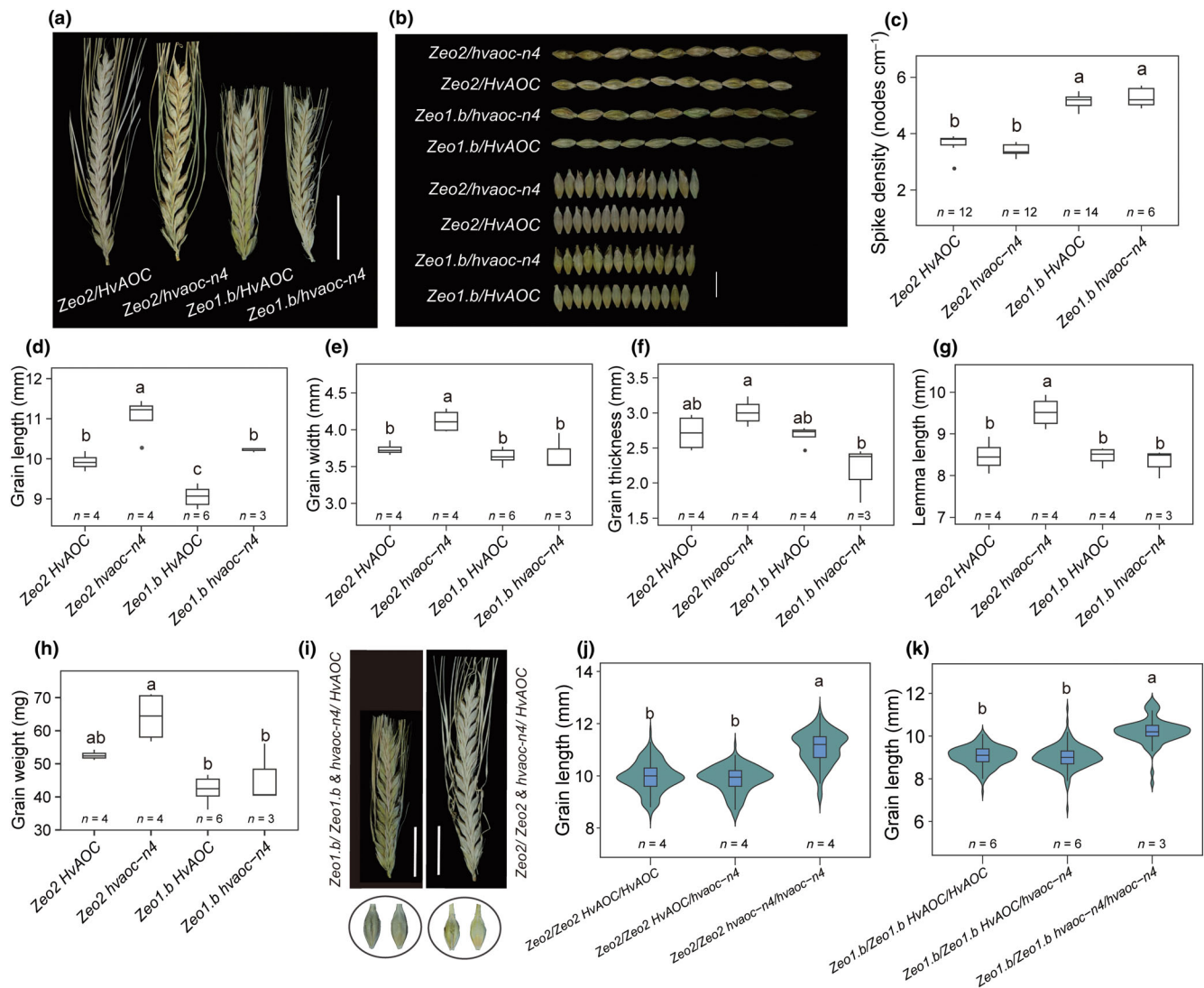


Fig. 4 Grain traits and lemma length in *Zeo1.b* × *hvaoc-n4* F3 siblings in barley. (a, b) Mature spike and grain morphology of *Zeo2/HvAOC* (*Zeo2/Zeo2 HvAOC/HvAOC*), *Zeo2 hvaoc-n4* (*Zeo2/Zeo2 hvaoc-n4/hvaoc-n4*), *Zeo1.b hvaoc-n4* (*Zeo1.b/Zeo1.b hvaoc-n4/hvaoc-n4*) and *Zeo1.b HvAOC* (*Zeo1.b/Zeo1.b HvAOC/HvAOC*). Bars: (a) 3 cm; (b) 10 mm. (c–h) Quantification of spike and grain morphological traits, including spike density (c), grain length (d), grain width (e), grain thickness (f), lemma length (g) and grain weight (h). The lower and upper box edges represent the first and third quartiles, the horizontal lines within the box indicate the median and the lower and upper whiskers denote the minimal and maximal values within 1.5× interquartile range, respectively, while points indicate outliers beyond this range. $n \geq 3$ per genotype. Different lowercase letters indicate significant differences ($P < 0.05$, one-way ANOVA, Tukey's HSD test). (i) Spike and grain morphology of *Zeo2/Zeo2 hvaoc-n4/HvAOC* and *Zeo1.b/Zeo1.b hvaoc-n4/HvAOC* at mature stage. Bar, 3 cm. (j, k) Grain length of *HvAOC/HvAOC*, *hvaoc-n4/HvAOC* and *hvaoc-n4/hvaoc-n4* both with *Zeo2/Zeo2* and *Zeo1.b/Zeo1.b* at mature stage. The lower and upper box edges within violin plot represent the first and third quartiles, the horizontal lines within box indicate the median and the lower and upper whiskers denote the minimal and maximal values within 1.5× interquartile range, respectively. $n \geq 3$ per genotype, with 178, 176 and 119 grains measured for *Zeo2/Zeo2 HvAOC/HvAOC*, *Zeo2/Zeo2 hvaoc-n4/HvAOC* and *Zeo2/Zeo2 hvaoc-n4/hvaoc-n4*, 190, 186 and 86 grains measured for *Zeo1.b/Zeo1.b HvAOC/HvAOC*, *Zeo1.b/Zeo1.b hvaoc-n4/HvAOC* and *Zeo1.b/Zeo1.b hvaoc-n4/hvaoc-n4*, respectively. Different lowercase letters indicate significant differences ($P < 0.05$, one-way ANOVA, Tukey's HSD test).

DNA in apoptotic cells with fluorescein-12-dUTP. We first observed TUNEL signal positive nuclei in Golden Promise caryopses at 6 DAP, mostly localized to regions laterally adjacent to the FVBs; by 10 DAP, the TUNEL signal localized at the upper FVB margins (Fig. 5j). By contrast, *hvaoc-n4* 6 DAP caryopses exhibited 64.9% fewer TUNEL signal spots adjacent to the FVB compared to Golden Promise (Table S6) while TUNEL-positive nuclei persisted in the surrounding pericarp at 10 DAP. Although

the distribution of TUNEL-positive nuclei in the NP region appeared similar between *hvaoc-n4* and WT by 10 DAP, Golden Promise showed more TUNEL signal spots (Fig. 5j; Table S6). Fewer TUNEL-positive nuclei in the VB and FVB of *hvaoc-n4* compared to Golden Promise suggests less degeneration in these tissues which marries well with their larger area in grain sections. Taken together, *HvAOC*, and accordingly JA, may be required for timely PCD in maternal vascular tissues.

To learn how internal features controlled by HvAOC earlier in grain development are reflected in mature, dried grain, we used X-ray micro computed tomography (μ CT) scans of mature, dried Golden Promise and *hvaoc-n4* grain ($n = 5$ per genotype). We found no difference in embryo, endosperm or aleurone volume,

despite the expected overall increase in *hvaoc-n4* grain length (Fig. S9). However, we detected clear changes under the hull in the central ventral region in a feature called the crease (Fig. 5k). The crease is a depression observed in some Triticeae, including barley and wheat, over the earlier vascular strands and NP

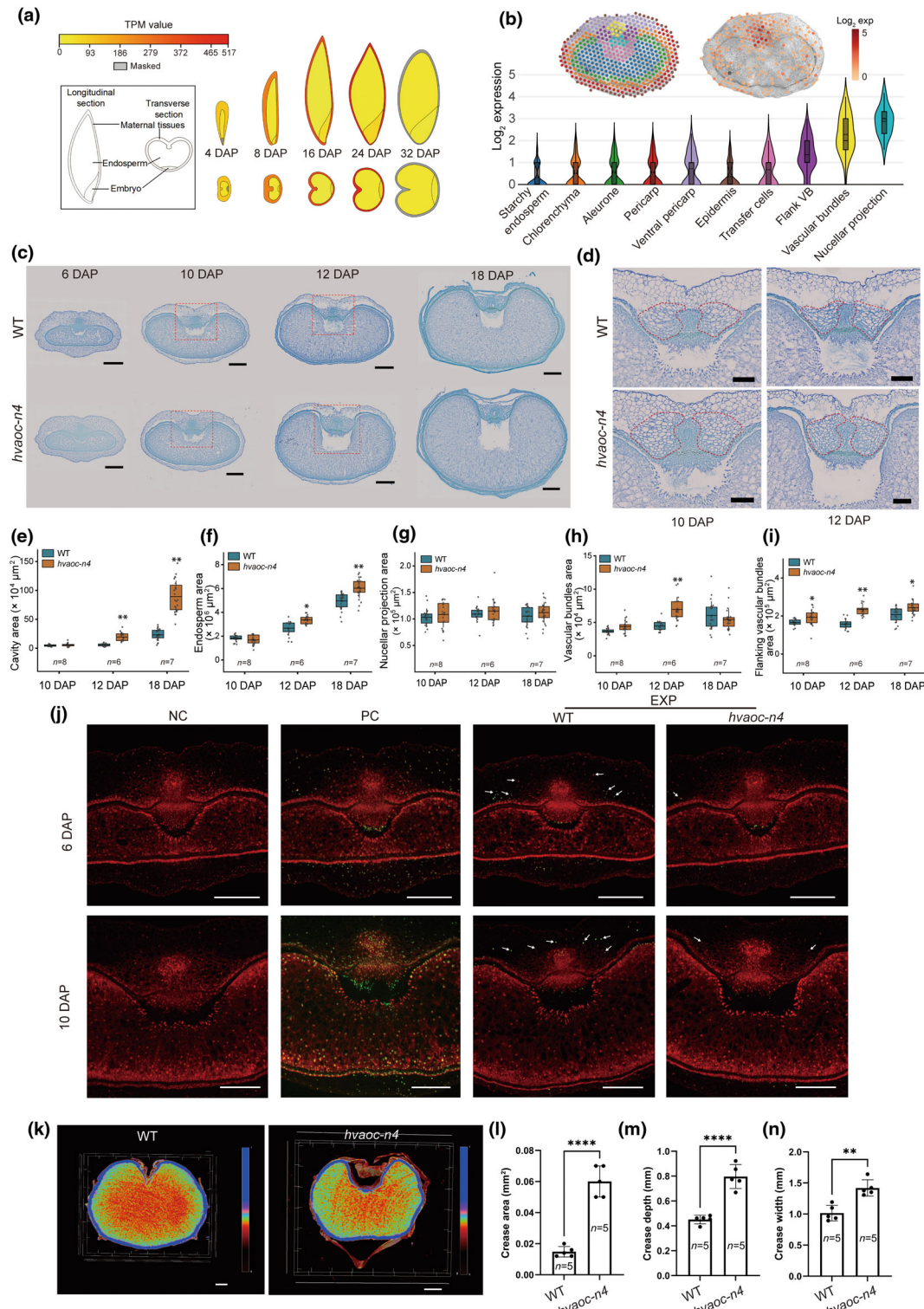


Fig. 5 *HvAOC* expression and influence on grain tissue development in barley. (a) Transcript levels of *HvAOC* detected in parental tissues, endosperm and embryo at different days after pollination (DAP), visualized from BAR-ePlant Barley V3 (<https://bar.utoronto.ca/>). Colours represent mean TPM values ($n = 3$) according to the heat map scale. Grey colour of parental tissues at 32 DAP indicates an expression level over 100% of their SD. TPM, transcripts per million. (b) Spatial expression pattern of *HvAOC* at 10 DAP in different grain tissues revealed by spatial transcriptomics. Violin plots show expression in spots assigned to different grain tissues. Grain image on left shows tissues and image on right shows *HvAOC* distribution. (c, d) Grain transverse sections of Golden Promise wild-type (WT) and *hvaoc-n4* at 6, 10, 12 and 18 DAP stained with toluidine blue. Red box indicates region at higher magnification in (d). Bars, 500 μm . (d) Magnified images of the nucellar projection (NP), vascular bundles (VB), regions flanking the vascular bundle (FVB) and cavity from sections shown in (c). Red dashed lines indicate the FVB region. Bars, 200 μm . (e–i) IMAGEJ quantification cavity (e), starchy endosperm (f), nucellar projection (g), vascular bundles (h) and regions flanking vascular bundles (i) area. Data were tested using Student's *t*-test, *, $P < 0.05$; **, $P < 0.01$. $n \geq 6$ per genotype, with three grain sections measured per individual as technical replicates. Grey dots in (e–i) represent one tissue area measurement. The horizontal line represents the mean, the bars represent SD, and the lower and upper edges of the box represent the first and third quartiles, respectively. The dots represent individual technical replicates. Each biological replicate was measured using three technical replicates. (j) Detection of programmed cell death by TUNEL assay in 6 and 10 DAP caryopsis sections. Green fluorescence indicates TUNEL-positive nuclei; white arrows mark TUNEL signals at the edge of the FVB in WT. Dashed boxes presented as magnified insets in the right most column. Red fluorescence shows propidium iodide counterstaining. EXP, experimental treatment; NC, negative control; PC, positive control. Bars, 400 μm . (k) Micro computed tomography (μCT) transverse slice from the middle of mature WT and *hvaoc-n4* grain. Bars, 500 μm . Colour bar right of image indicates material density. (l–n) Quantification of WT and *hvaoc-n4* grain crease area (l) depth (m) and width (n) from transverse sections. Dots indicate individual biological replicates. $n = 5$ grains per genotype. ****, $P < 0.0001$; **, $P < 0.01$.

(Hands *et al.*, 2012). The crease in *hvaoc-n4* was significantly enlarged compared to Golden Promise, showing 390% increased area, 140% increased width and 177% increased depth (Fig. 5l–n) based on measurements from transverse slices in the middle of the grain. The crease likely represents the collapse of degraded maternal tissues into the earlier formed cavity, suggesting that the larger cavity in developing *hvaoc-n4* caryopses compared to Golden Promise persists throughout grain maturation and drying.

Loss of *HvAOC* function influences gene expression associated with sugar transport, endosperm development, cell death and defence signalling

To determine how loss of *HvAOC* function influences GE during grain development, we performed comparative RNA-seq on 6 and 12 DAP caryopses harvested from Golden Promise and *hvaoc-n4* mutants. We detected 2055 and 406 DEGs between genotypes at 6 and 12 DAP, respectively, with 52 DEGs shared between stages (Fig. 6a; Table S7). Plotting spatial patterning of DEGs revealed highest concentration in parental transfer tissues in 6 DAP grain but also DEGs in filial tissues such as the endosperm and transfer cells (Fig. 6b). DEGs at 6 DAP were enriched for several GO terms (Fig. 6c; Table S8), such as seed developmental processes (GO:0009793), which included genes encoding Sugars Will Eventually be Exported Transporters (SWEETs; Fig. 6e): *hvaoc-n4* 6 DAP caryopses showed 4.6-fold increased *HvSWEET14a* (HORVU.MOREX.r3.1HG0010640) and 2.78-fold increased *HvSWEET14b* (HORVU.MOREX.r3.6HG0540540) expression, the latter gene known to promote sucrose transport from maternal tissues into the grain (Radchuk *et al.*, 2023). Other GO-enriched terms included primary meristem (GO:0010072) (TPLs and PHB) and inflorescence development (GO:0048281), including auxin transporter BIG (HORVU.MOREX.r3.5HG0469420) and ERECTA kinases (Fig. 6e; Table S8). We also detected downregulated DEGs under enriched terms for starch catabolism (GO:0005983) and beta-glucan synthesis (GO:0006075). While not associated with an enriched GO-term, the barley gene

(HORVU.MOREX.r3.6HG0624320) was strongly downregulated in *hvaoc-n4* and is predicted to encode a class I MADS-box-encoding gene orthologous to *OsMADS87* which is epigenetically repressed to promote timely endosperm cellularization (Folsom *et al.*, 2014; Chen *et al.*, 2016; Fig. 6e; Table S7). By 12 DAP, enriched terms included sucrose synthesis (GO:0005986), representing several upregulated fructose-1,6-bisphosphatases, and gluconeogenesis (Fig. 6d; Table S8). We also detected enrichment GO terms such as response to water (GO:0009414) and vacuolar processing (GO:0006624), which included genes encoding vacuolar processing enzymes which were downregulated in *hvaoc-n4*, such as *VPE2c* (HORVU.MOREX.r3.2HG0184580) downregulated 1.88-fold and *VPE2b* (HORVU.MOREX.r3.2HG0184570) downregulated 1.74-fold (Fig. 6e), the latter gene previously shown as expressed in the NP and associated with PCD (Radchuk *et al.*, 2011). Cereal endosperm, including that in barley, is O₂-deficient (Rolletschek *et al.*, 2004). Interestingly, the enriched term response to hypoxia (GO:0071456) represented DEGs all upregulated in *hvaoc-n4* mutant (Table S7), while one of the most highly upregulated genes in *hvaoc-n4* at 12 DAP encodes a AP2-ERF transcription factor (HORVU.MOREX.r3.2HG0112960), member of a family known to mediate hypoxia-related GE in Arabidopsis (Yang *et al.*, 2011). While no SWEET-encoding genes contributed to GO-enriched terms at 12 DAP, the *hvaoc-n4* mutant showed 1.5-fold upregulation of *HvSWEET14a* (HORVU.MOREX.r3.1HG0010640), similar to 6 DAP, and a 1.76-fold upregulation of *HvSWEET11b* (HORVU.MOREX.r3.7HG0684580; Table S7), reported to be highly expressed in nucellar parental tissues and to transport sucrose and cytokinin and promote endosperm cell number (Radchuk *et al.*, 2023). In addition to *HvSWEET14a*, the most highly upregulated DEGs shared between 6 and 12 DAP in *hvaoc-n4* mutants included Pathogenesis-related and TGA transcription factors, defence genes which could be related to changes in PCD and/or differences in cavity formation (Table S7). Another shared DEG encodes gibberellin oxidase 20 (HORVU.MOREX.r3.4HG0331680), a key biosynthetic step in producing the growth-promoting gibberellin phytohormone that was

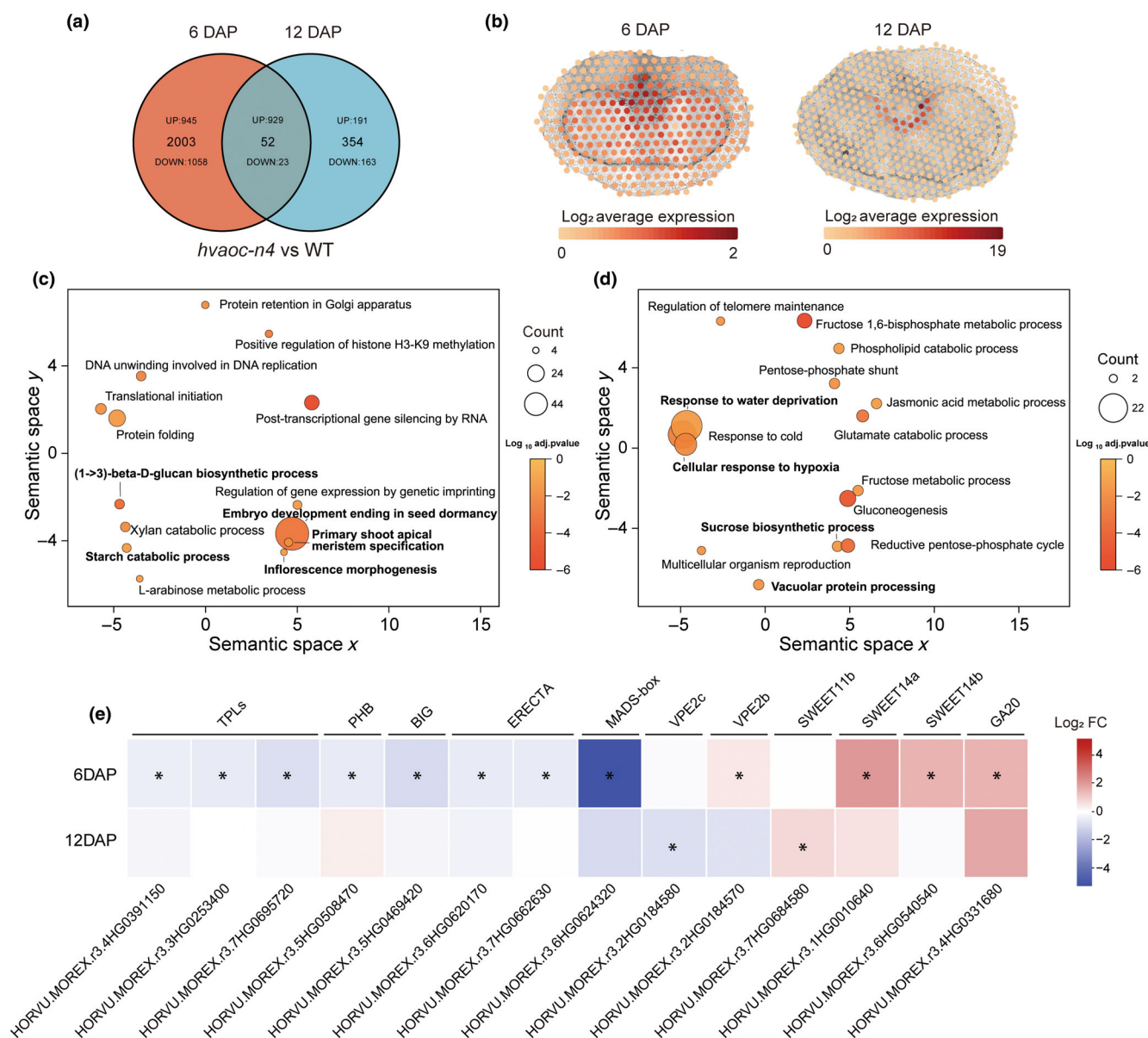


Fig. 6 Transcriptomic alterations in the *hvaoc-n4* mutant during early grain development in barley. (a) Venn diagram showing differentially expressed genes (DEGs) in *hvaoc-n4* compared with wild-type (WT) at 6 and 12 d after pollination (DAP). (b) Expression landscape of DEGs showing the average expression intensity of DEGs at 6 and 12 DAP mapped to 7 DAP and 10 DAP grain. (c, d) Semantic similarities between significantly enriched Gene Ontology (GO) categories in DEGs in *hvaoc-n4* compared with WT at (c) 6 DAP and (d) 12 DAP. Bubbles represent GO terms whose closeness corresponds with semantic similarity. Bubble colours represent P -values and bubble sizes represent the number of DEGs carrying this term. (e) Heatmaps based on expression fold changes of representative DEGs related to grain development in *hvaoc-n4* compared with WT at 6 and 12 DAP. DEGs with expression $|\log_2$ fold change| ≥ 0.5 and a Benjamini–Hochberg adjusted P -value ≤ 0.05 are highlighted by asterisks.

upregulated 2.8-fold at 6 DAP and 3.2-fold at 12 DAP in *hvaoc-n4* mutants. Additionally, the barley ortholog (HORVU.MOREX.r3.2HG0174520) of the rice *DWARF4* gene, which encodes a cytochrome P450 essential for brassinosteroid biosynthesis and which promotes grain length (Tanabe *et al.*, 2005), was 17-fold elevated in the mutant. Taken together, GO enrichment and individual genes differentially expressed in *hvaoc-n4* caryopses, especially the *HvSWEET*, *HvVPE*, phytohormone and defence genes, suggest changes in parental tissue development and

increased nutrient flow which may contribute to larger endosperm in *hvaoc-n4* caryopses.

JA biosynthetic and signalling pathway genes show distinctive expression in the grain spatial transcriptome

We also examined genes encoding all steps in the JA biosynthetic pathway across the spatial transcriptome at 7 DAP (Fig. S10, Table S9). Within multigene families, selected individual genes

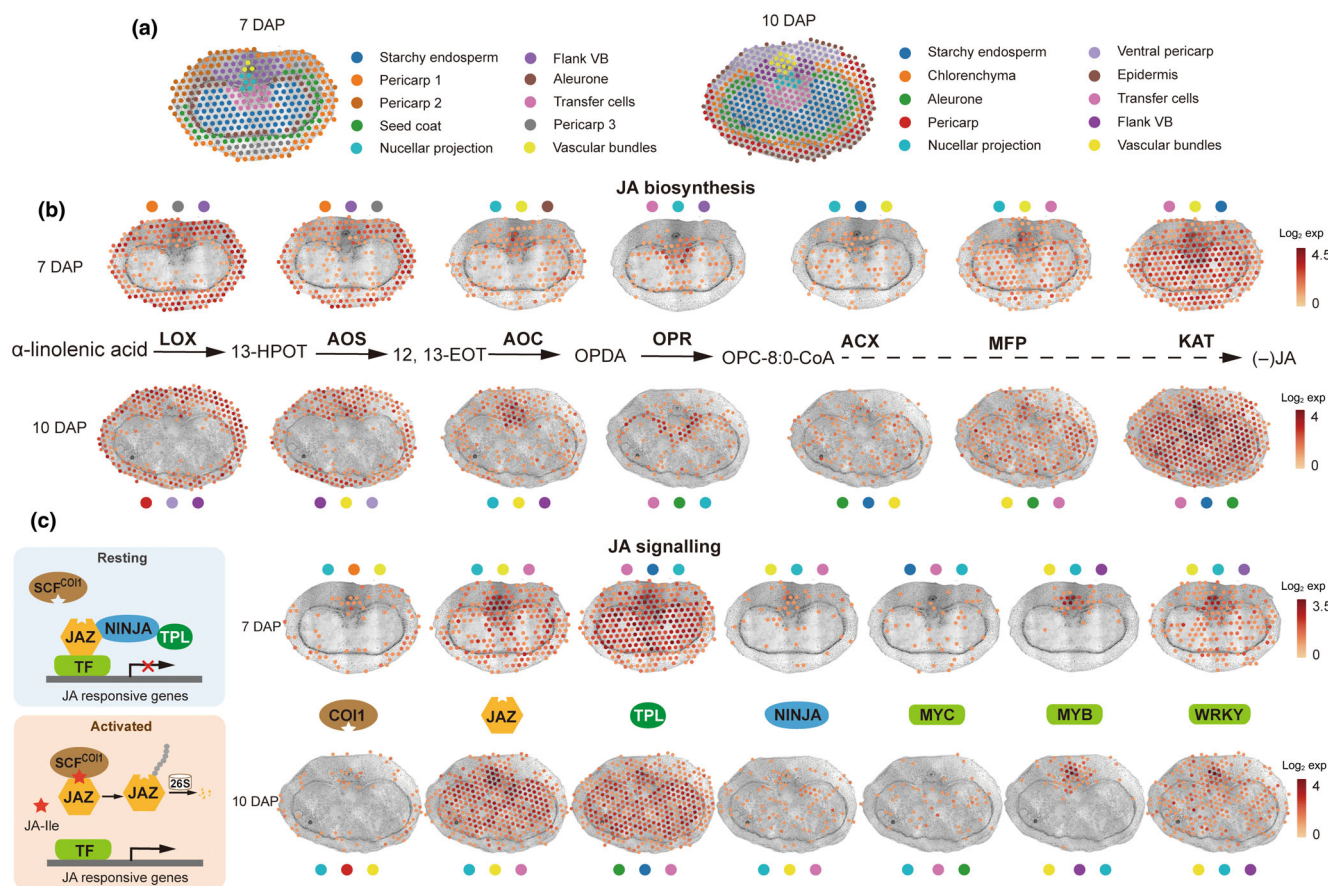


Fig. 7 Spatial expression patterns of JA biosynthesis and signalling genes in barley grains. (a) Tissue annotation of spatial transcriptomic sections from barley grains at 7 and 10 d after pollination (DAP). Tissues were generated using K-means pipeline and the cluster number was set to 10. (b) Spatial expression maps of key enzymes in the JA biosynthetic pathway at 7 and 10 DAP. Genes involved in successive steps from α -linolenic acid to (-)-JA, including LOX, AOS, AOC, OPR, ACX, MFP and KAT, are shown. The spatial heatmap shows the results of the highest-expressed gene of each gene family based on the RNA-seq expression data. 13-HPOT, 13(S)-hydroperoxyoctadecatrienoic acid; 12,13-EOT, 12,13(S)-epoxyoctadecatrienoic acid; OPDA, (9S,13S)-12-oxo-phytodienoic acid; OPC 8:0-CoA, 3-oxo-2-(*cis*-2'-pentenyl)-cyclopentane-1-octanoic acid CoA. LOX, lipoxygenase; AOS, allene oxide synthase; AOC, allene oxide cyclase; OPR, OPDA reductase; ACX, acyl-CoA oxidase; MFP, multifunctional protein; KAT, 3-ketoacyl-CoA thiolase. (c) Schematic representation of the JA signalling pathway (left), illustrating the JAZ–NINJA–TPL repressor complex in the resting state and COI1-mediated JAZ degradation upon JA-Ile activation. Spatial expression maps (right) display the distribution of major JA signalling components at 7 and 10 DAP, including COI1, JAZ, TPL, NINJA and transcription factors (MYC, MYB and WRKY domain factors). COI1, CORONATINE INSENSITIVE1; JAZ, JASMONATE ZIM-DOMAIN; TPL, TOPLESS; NINJA, NOVEL-INTERACTOR-OF-JAZ. Spatial heatmaps in (b) and (c) represent log₂-transformed UMI counts across tissue domains. Coloured circles above and below each heatmap in (b) and (c) denote the top 3 tissue domains with the highest expression for the corresponding gene. For key JA biosynthesis enzyme genes and signalling components, only the members of the gene family with the highest expression were used to generate the heatmap, the average expression heatmaps of whole gene family are shown in Supporting Information Figs S10 and S11.

showed distinctively higher expression compared to other members suggesting more important roles in JA production during grain development. The barley orthologs of *LOX* and *AOS* genes involved in early steps in JA biosynthesis were enriched in maternal tissues: for instance, transcripts of *HvLOX* genes (HORVU.MOREX.r3.5HG0509200, HORVU.MOREX.r3.4HG0335790 and HORVU.MOREX.r3.6HG0614350) and the *HvAOS* genes (HORVU.MOREX.r3.4HG0394980 and HORVU.MOREX.r3.5HG0513120) appeared more frequent in the pericarp and vascular regions while *HvAOC* localized to pericarp VBs and the NP (Figs 7, S10). Interestingly, the most highly expressed *HvAOS* gene HORVU.MOREX.r3.4HG0394980 was downregulated in the *hvaoc-n4* mutant, as were several other biosynthetic

genes (Table S7) possibly suggesting negative feedback. Gene families associated with later steps in JA biosynthesis were more broadly expressed (Figs 7, S10). Of particular interest were *HvOPR* genes, HORVU.MOREX.r3.7HG0726360, HORVU.MOREX.r3.4HG0331730 and HORVU.MOREX.r3.2HG0170590, which were most strongly expressed in VBs, NP and filial transfer cells, respectively (Figs 7, S10). We also examined genes encoding key signalling components including the COI1 receptor, JAZ inhibitors and TPL and NINJA co-factors (Fig 7, Table S9). At 7 DAP, *HvCOI1* transcripts were clearly enriched in maternal tissues, while *JAZ* transcripts were similarly expressed in maternal tissues, especially the NP, but with some minor expression in filial tissues (Figs 7, S11). On the other hand,

HvNINJA transcripts were most highly expressed in transfer cells and moderately expressed in all tissues with the while *HvTPLs* were highly expressed in all tissues. At 10 DAP, JA biosynthesis genes were similarly expressed as in 7 DAP with early steps were still mostly limited to maternal tissues and later steps more broadly expression. However, while *HvCOI1* expression pattern seemed little changed at 10 DAP compared to 7 DAP, we detected a shift in the *HvJAZ* gene, HORVU.MOREX.r3.2HG0203510, which was little expressed at 7 DAP but strongly upregulated in all tissues at 10 DAP with highest expression in VBs and the NP. We detected other less dramatic changes in the *TPL* and *NINJA* genes (Fig. S11). Interestingly, we detected little expression and no tissue specificity for *MYC2*, the classic TF downstream of JA signalling, which was also absent from the DEGs. However, transcription factors, including MYBs and other bHLHs mediate JA signalling in other contexts (Huang *et al.*, 2017, 2023), which could also be the case for barley grain, and likely work through a JA-sensitive association with JAZ proteins to determine their activity.

Discussion

We tested our hypothesis that JA regulates vegetative and reproductive growth in barley by editing *HvAOC*, a single-copy gene encoding an enzyme acting early in JA biosynthesis. We characterized two Cas9-free lines with different predicted loss-of-function mutations in *HvAOC*. Our results suggest that *HvAOC* function is important to restrict coleoptile and early seedling growth and delays flowering based on time to anthesis (Fig. 1). Two rice mutants with defective *AOC* alleles were reported to show enhanced coleoptile growth and earlier heading, suggesting these roles are conserved between rice and barley. However, the rice mutants also formed elongated floret hulls (Riemann *et al.*, 2013) which we did not observe in *hvaoc* mutants in the Golden Promise background. Nonetheless, *hvaoc* mutants containing mixed Bowman and Golden Promise backgrounds developed longer lemmas compared to siblings with the *HvAOC* wild-type allele (Fig. 4), suggesting that other genes may interact with *HvAOC* to regulate this phenotype.

The splayed lemmas observed in *hvaoc* mutants derived from longer and deeper caryopses which prised open the floret hulls (Figs 1k, 2a). Caryopses dimensions differed between Golden Promise and *hvaoc-n4* early in development, and corresponded to increased maternal tissues, especially of the regions FVB, as well as increased cavity and endosperm area (Fig. 5). In Arabidopsis, JA signalling represses integument cell proliferation and ovule size (Hu *et al.*, 2021). In barley, *hvaoc* mutant integument and pericarp layers must be larger as they enclose a larger seed. Since our data shows pericarp epidermis cells did not change in size, we suggest that larger tissues reflect increased cell proliferation. Our previous work showed that *HvAP2* function restricted pericarp cell number and limited integument layer number, as well as regulating the rate and extent of maternal tissue degradation (Shoosmith *et al.*, 2021). Thus, we speculate that the gain of *HvAP2* function in *Zeo1.b* mitigated *hvaoc*-driven increases in grain length in double mutants by partially suppressing

enhanced proliferation in maternal layers due to the loss of *HvAOC*.

In rice, *coi* receptor mutants and overexpression of the JA signalling repressor *OsJAZ11* increases grain width and weight, indicating a role for JA signalling in limiting grain growth (Mehra *et al.*, 2022). In vitro assays detected a direct protein interaction between *OsJAZ11* and *OsMADS29*, a key regulator of NP differentiation and death, suggestive of a role for JA signalling in parental tissues, especially the NP, to promote maternal tissue differentiation and degradation. Our anatomical, TUNEL assay and transcriptomic results also support a role for JA in promoting maternal tissue degradation in barley, potentially upstream of a JAZ11-MADS29 module. While parental death is important for filial tissue growth (Domínguez & Cejudo, 2014), increased persistence and size of regions FVB in *hvaoc* mutants correlated with increased cavity and endosperm area (Fig. 5), as well as transcriptomic evidence for enhanced nutrient transport (Fig. 6). As vascular tissues transport nutrients and sugar metabolites from parental reserves into grain (Olsen, 2004), we speculate that larger transport tissues and/or cavity volumes increase nutrient flow, consistent with previous suggestions that enlarged cavities facilitate assimilate transport and enhance endosperm growth (Radchuk *et al.*, 2021). Furthermore, since the endosperm itself constitutes an O₂ diffusion barrier (Langer *et al.*, 2023), the larger endosperm volume and increased starch biosynthesis could increase the level of hypoxia, as reflected in the hypoxia-related DEGs upregulated *hvaoc*. Hypoxia is a state important for nutrient transit and starch metabolism (Borisjuk & Rolletschek, 2009) so increased endosperm volume may feed forward to further reduce O₂ diffusion. A recent study in maize showed that expression of a monocot-specific homolog of Inducer of CBF Expression 1 (*ZmICE1*) in the endosperm specifically downregulated JA synthesis and defence genes in the aleurone to promote starch synthesis, suggesting that regulatory units within the endosperm modulate defence vs growth through JA. We note that *HvAOC* is expressed in the aleurone at 7 DAP, in addition to maternal tissues, so could also control JA production in filial transfer tissues. Nonetheless, our evidence of spatial patterns of JA biosynthesis and signalling components coupled with segregation patterns indicate a central role for maternal tissue in generating JA and/or precursors or other products which themselves could move directly to or otherwise drive signalling in both maternal and filial tissues to change grain dimensions (Fig. 7).

We propose a mechanism aligned with the classic growth vs defence paradigm (Guo *et al.*, 2018), wherein JA limits maternal growth and accelerates its degeneration and nutrient delivery to regulate final grain parameters. As such, manipulating the JA pathway presents a possible route to increased grain size by lengthening the window through which parental resources can be transported to the filial tissues. Moreover, altering the rate of cell death or senescence during grain development could also help increase resiliency of grain fill under stressful conditions. Regulating jasmonate metabolism may promote wheat growth under elevated temperatures (Zhu *et al.*, 2021). It will be important to learn whether similar mechanisms also work in the grain under warmer conditions.

Acknowledgements

We are grateful for support from the University of Dundee Imaging Facility as well as Imaging Technologies at the James Hutton Institute (JHI) and Sheffield Hallam University. We also acknowledge support from the Barley Genetics group at JHI. We also acknowledge services and support from the Genome Technology Lab at JHI. This research was funded by Biotechnology and Biological Sciences Research Council grant BB/W003074/1 awarded to SMM. JL was supported by the China Scholarship Council and the University of Dundee. JG and JS were supported by the University of Dundee and BB/W003074/1. OD is supported by a Sheffield Hallam and La Trobe University PhD scholarship. This research was facilitated by a UK-Australia Partnership Award BB/V018299/1 awarded to SMM. XY is supported by the Mortlock Bequest, Adelaide University. The authors acknowledge the instruments and expertise of Microscopy Australia (ROR: 042mm0k03) at Adelaide Microscopy, University of Adelaide, enabled by NCRIS, university and state government support (Percival-Alwyn *et al.*, 2024).

Competing interests

None declared.

Author contributions

SMM, JL and LL designed the research. JL, JS, MN, MK-F, RH, EL, OD, CT, TD and SMM performed the research. WW and JG developed experimental techniques. SMM, JL, CT, TD, OD and LL analysed experimental data. NA and NB advised on μ -CT. GZ provided facilities. SMM and JL wrote the manuscript. XY and MRT shared VISIUM data and methods. All authors had input into the manuscript.

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Data availability

All raw RNA-seq data from this study has been deposited at the European Nucleotide Archive (ENA) under project accession PRJEB123707. Germplasm available upon request. Sequences

for gene models are found in Ensemble using the gene model IDs provided in this paper: [Hordeum_vulgare-Ensembl Genomes 62](#).

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Phylogenetic relationships and motif comparisons of AOC proteins from monocots and eudicots.

Fig. S2 Allelic variation and haplotype structure of *HvALLENE OXIDASE CYCLASE*.

Fig. S3 Expression patterns of *HvALLENE OXIDASE CYCLASE* in different barley organs.

Fig. S4 LC-MS plots for JA, JA-Ile and OPDA detected in *hvaoc-n4* and Golden Promise seedling tissues.

Fig. S5 Germination rate of Golden Promise (WT), *hvaoc-n4* and *hvaoc-n7* grain.

Fig. S6 Spike-related phenotypes of Golden Promise (WT) *hvaoc-n4* and *hvaoc-n7*.

Fig. S7 Plant height of *Zeo1.b* x *hvaoc-n4* F3 siblings.

Fig. S8 Grain parameters in *hvaoc-n4/+* and Golden Promise (WT) individuals.

Fig. S9 Micro Computed Tomography of Golden Promise (WT) and *hvaoc-n4* mature grains.

Fig. S10 Tissue-specific expression heatmaps of JA biosynthesis genes in developing barley grains.

Fig. S11 Tissue-specific expression heatmaps of JA signalling components in developing barley grains.

Table S1 Germplasm used in this study.

Table S2 Guide sequences for gene editing.

Table S3 Primers used for genotyping.

Table S4 Micro Computed Tomography Scanning Parameters.

Table S5 Expected and observed segregation of *HvAOC* and *HvAP2* alleles in *Zeo1.b* x *hvaoc1-n4* F2 population and F3 siblings.

Table S6 TUNEL-positive spot counts of *hvaoc-n4* and Golden Promise at 6 and 12 d after pollination (DAP).

Table S7 Differentially expressed genes in *hvaoc-n4* compared to Golden Promise caryopses.

Table S8 Gene Ontogeny enrichment of differentially expressed genes.

Table S9 Heat map values of JA biosynthesis and signalling pathway.

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