Structure-activity relationships and crucial mechanisms of the coumarin family as germination inhibitors

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Abstract

Members of the coumarin family, including coumarin and its derivatives, are molecules produced by various higher plants, such as cherry trees, and are noted for their diverse physiological activities. Notably, these compounds serve as germination inhibitors, which is considered their primary role. However, the structural basis underpinning the germination-inhibitory effects of the coumarin family is not well understood. Our research investigated the structure-activity relationships using coumarin analogs and revealed that the benzene ring and ester bond within the coumarin skeleton play a crucial role in germination inhibition. We also identified a correlation between the topological polar surface area (TPSA) and the inhibitory effects on germination. Furthermore, while the physiological effects of coumarin on seeds prior to germination have been reported variably, the mechanisms of germination inhibition remain largely unknown. Using RNA-Seq analysis, we have discovered a novel mechanism that is pivotal for germination inhibition, where coumarin suppresses the expression of genes associated with cellulose fiber reassembly. This study holds significant importance in understanding how the coumarin family contributes to plant competition in nature and promises future contributions to the agricultural field through the development of novel growth regulation methods.

32 **Keywords:** Natural inhibitors, Coumarin structure, Cheminformatics, RNA-Seq, Cell wall

33 reconfiguration, Ribosome biogenesis

34

Introduction

Coumarin belongs to the benzopyrone family and is produced by various higher plants, including the cherry tree[1,2]. In Japan, there is a tradition of enjoying the aroma of "sakurayu," literally "cherry blossom tea," and "sakuramochi," a sweet made with mochi (Japanese rice cake) and preserved cherry leaves, which primarily derives its scent from coumarin[3]. Thus, coumarin has a deep association with Japanese traditional culture. Furthermore, not only is coumarin itself produced by some plants, but a variety of coumarin derivatives are also known to be naturally synthesized[2], and others are artificially created. For simplicity, both coumarin and its derivatives will be collectively referred to as the "coumarin family." Members of the coumarin family are known for their diverse physiological activities and are particularly noteworthy in medicine, where, for example, warfarin, a representative member of the coumarin family, is used as an anticoagulant to prevent thromboembolic disorders[4]. Additionally, artificially synthesized coumarin derivatives are gaining attention as potential candidates for new anticancer agents[5]. Thus, members of the coumarin family are gaining interest as compounds with potential applications in pharmaceuticals and other fields.

However, the primary role of the coumarin family in plants is to inhibit the growth of surrounding vegetation through their production. The main mechanism by which members of the coumarin family

inhibit growth is through a strong inhibitory effect on seed germination[6]. Regarding the germination inhibition by members of the coumarin family, it is unclear which structures are important, as well as how and where these compounds impact the seeds.

One of the unresolved questions is which structural elements of the coumarin family members are critical for germination inhibition, namely, the correlation between the molecular structures of various coumarin family members and their germination inhibitory effects. Mayer et al. conducted a comparison and reported that structural modifications to coumarin do not destroy the germination inhibitory effect but only partially attenuate it[7]. This suggests that the germination inhibition effect is conserved among many members of the coumarin family. On the other hand, coumarin derivatives with more potent phytotoxic effects than coumarin itself have been discovered[8], indicating a need for further research into the impact of such modifications.

Another unresolved issue is where and how coumarin family members influence seeds to achieve germination inhibition. A critical factor in determining where coumarin family members affect seeds is their impact on the seed coat. As the rupture of the seed coat is crucial for germination, there is a possibility that the members of the coumarin family act on the seed coat they first come into contact

with. Recent studies have shown that a thick and low water-permeable seed coat plays a role in negatively regulating the initiation of seed germination[9]. Interestingly, Aliotta et al. demonstrated that the removal of the seed coat significantly diminishes the effect of coumarin[10]. Moreover, the interaction of coumarin family members with the seed coat may alter its properties, potentially changing the water absorption rate of the seed. Thus, while there is a likelihood that coumarin family members act primarily on the seed coat as described above, it is also necessary to verify whether they penetrate the seed coat and act inside it.

A critical aspect of understanding how the coumarin family members influence seed germination is their effect on cellular processes. Several studies have explored this mechanism, with the studies by Chen et al. particularly notable[11,12]. They demonstrated that coumarin negatively regulates the expression of the abscisic acid (ABA) metabolic enzyme genes (OsABA8' ox2/3) and reduces the levels of gibberellin A4 (GA_4). Furthermore, coumarin also suppresses the accumulation of reactive oxygen species (ROS) and the expression of metabolic enzymes such as superoxide dismutase and catalase. These outcomes physiologically substantiate the delay in seed germination induced by coumarin. Omics analyses have greatly contributed to the elucidation of the mechanisms behind germination inhibition[13,14]. Araniti et al. conducted metabolomic analyses, revealing that coumarin

86 extensively affects the seed's metabolic profile, impacting amino acid metabolism, the citric acid
87 (TCA) cycle, and aminoacyl-tRNA biosynthesis[13]. A similar analysis by Zhang et al. is consistent with
88 Araniti et al.'s findings[14]. Additionally, Zhang et al. conducted transcriptomic analyses 72 hours after
89 coumarin addition, showing that coumarin influences the expression of genes involved in amino acid
90 metabolism, the TCA cycle, and protein translation. The outcomes of these transcriptomic analyses
91 are invaluable. However, given that they were conducted 72 hours after the addition of coumarin, they
92 may reflect gene expression after a range of changes had already occurred. Many previous studies may
93 not have clearly identified the final targets of coumarin-induced germination inhibition, implying they
94 did not account for the cellular structure changes expected during inhibition of germination.

95

96 In this study, we addressed the unresolved questions we had raised. To identify the structures critical
97 for germination inhibition, we explored the structure-activity relationships and pinpointed the
98 responsible substructures for germination inhibition effects using various coumarin analogs. We also
99 compared the germination inhibition effects with various molecular indices, such as molecular volume
100 and polar surface area, as predicted by cheminformatics. To investigate where coumarin affects seeds,
101 we measured the changes they induce in the seed coat structure and the seed's water uptake. In addition,
102 we examined the localization of coumarin by studying their absorption spectrum. Finally, to assess the

103 early effects of coumarin, we performed a transcriptomic analysis 24 hours after the addition of

104 coumarin.

105

Results

The impact of coumarin substructures on germination inhibition

Our experimental investigation into the structure-activity relationship of coumarin (Fig. 1A) has revealed that the ester bond and benzene ring within its substructure are critical for germination inhibition, while the presence of a double bond is not essential. In this study, we tested the germination effects on clover seeds using compounds that have similar substructure to coumarin: α-pyrone, which lacks coumarin's benzene ring (Fig. 1B), β-tetralone, missing both the double bond and ester bond from coumarin's lactone ring (Fig. 1C), and 3,4-dihydrocoumarin, lacking only the double bond from the lactone ring (Fig. 1D). The germination rate for seeds with just water added (negative control) was 94%, and for those with coumarin added, it was 29%. The germination rates for seeds treated with α-pyrone and β-tetralone were 82% and 86%, respectively. These rates were significantly higher than those observed for seeds treated with coumarin and were only slightly lower than the negative control, albeit significantly so (Fig. 1E). These results demonstrate the importance of the ester bond and benzene ring in the substructure of coumarin for germination inhibition. On the other hand, the germination rate for seeds treated with dihydrocoumarin was 12%, which was significantly lower than the coumarin-treated seeds and the negative control. This indicates that the absence of the double bond in coumarin's substructure does not play a significant role in germination inhibition.

Prediction of germination inhibition effects by the coumarin family through TPSA (topological polar surface area)

Our experimental investigations into the germination inhibition effects of various members of the coumarin family revealed that the topological polar surface area (TPSA) is an effective predictor of germination inhibition. In addition to coumarin and 3,4-dihydrocoumarin, we used the compounds shown in Fig. 2A–D for our analysis. We calculated the molecular volume, molecular surface area, lipophilicity index LogP, and another lipophilicity index TPSA (Topological Polar Surface Area), for these substances (Supplementary Table 1) and examined the correlation between these values and the effects on germination inhibition. Our analysis detected no significant correlation between germination inhibition and molecular volume ($p=0.31 > 0.05$), surface area ($p=0.25 > 0.05$), or LogP ($p=0.26 > 0.05$) (Fig. 2E–G). However, we identified a significant correlation between germination inhibition and TPSA ($p=0.019 < 0.05$), with a high correlation coefficient ($r=0.8349$) (Fig. 2H).

Observation of changes in seed coats treated with coumarin

We investigated the possibility of coumarin acting on the outermost layer of the seed, the seed coat, to

139 inhibit germination and found no changes in the structure or permeability of the seed coat. Examination
140 of pea seed coats with a scanning electron microscope revealed a three-layered structure consisting of
141 palisade parenchyma, hourglass cells, and compressed parenchyma, regardless of whether coumarin
142 was administered (Fig. 3A). Furthermore, measurements of the overall thickness of the seed coat
143 indicated that both the coumarin-treated and control seeds had seed coats approximately 90 μm thick,
144 with no detectable difference. Similarly, measurements of the thickness of each tissue layer also did
145 not reveal any significant differences due to coumarin treatment (Fig. 3B). Additionally, when
146 measuring water absorption of the seeds, both the coumarin-treated and control seeds began to absorb
147 water immediately upon immersion. For both, the water absorption rate reached a near plateau after
148 about 12 hours, and thereafter, very little additional water was absorbed (Fig. 3C).

149

150 **Penetration of coumarin beyond the seed coat into the seed interior**

151 Based on the above results, we inferred that coumarin penetrates the seed coat and enters the interior
152 of the seed, which we then demonstrated. We measured the absorption spectrum of the liquid obtained
153 from crushed pea seeds from which the seed coat had been removed. The results showed a peak around
154 the wavelength of 270 nm, which corresponds to the absorption maximum of coumarin, only in the
155 extract from the coumarin-treated seeds (Fig. 3D).

156

157 **Transcriptomic impact of coumarin on seed germination**

158 Utilizing Arabidopsis, which has a well-established genetic profile, we examined the impact of
159 coumarin on seeds using RNA-Seq analysis. We found that coumarin notably alters the expression of
160 gene groups involved in protein translation and cellular elongation growth, which is mediated by the
161 reassembly of cellulose fibers. The RNA-Seq analysis compared gene expression in seeds treated with
162 coumarin for 24 hours with control seeds treated with water only. Three biological replicates for each
163 group resulted in a total of six samples for analysis, with an average of 3.9 Gbp of nucleotide sequences
164 obtained per sample. Out of 21,133 detected genes, 3,416 were identified as differentially expressed
165 genes (DEGs) (Supplementary Spreadsheets 1). Notably, gene groups coding for proteins that
166 constitute the large subunit of ribosomes, RPL (111 genes), and the small subunit, RPS (80 genes),
167 had their expression significantly suppressed by coumarin treatment (Fig. 4A and 4B, Supplementary
168 Spreadsheet 2 and 3). Many genes of the expansin family (EXPA2/3/4/8/9/15, ATEXPA1/10) also
169 exhibited a significant reduction in expression (Fig. 4C, Supplementary Table 2)

170

171 The results of the pathway analysis of the gene set enrichment analysis (GSEA) are presented in Table
172 1. In seeds treated with coumarin, a downregulation of gene expression was observed in "Ribosome"

173 and "Ribosome, eukaryotes," while an upregulation was noted in three pathways including
174 "glutathione-mediated detoxification II," "β-Oxidation," and "Jasmonic acid biosynthesis" (Fig. 5A).
175 Subsequent results of the GSEA Gene Ontology (GO) analysis are detailed (Fig. 5B–D, Supplementary
176 Spreadsheet 4). The analysis of GO Process analysis indicated that genes related to "sulfate
177 assimilation" and "response to toxic substances" were activated, while those involved in "ribosome
178 biogenesis," "translation," and "unidimensional cell growth" were inhibited (Fig. 5B). Furthermore,
179 the analysis of GO Component revealed that genes forming parts of "vacuoles" were activated, while
180 the expression of genes constituting "ribosomes" and the "cell wall" was suppressed (Fig. 5C).
181 Additionally, under GO Function, genes associated with "RNA binding" and "glutathione gamma-
182 glutamylcysteinyltransferase activity" showed increased expression (Fig. 5D).

183

184 From the association with expansins, we focused on the enrichment of "cell wall," "plant-type cell
185 wall," and "unidimensional cell growth" in the GO analysis (Supplementary Table 3). Analysis of these
186 enriched groups included many expansin family genes. Other genes involved in the metabolism of
187 xyloglucan, such as α-Xylosidase1 (XYL1) and the Xyloglucan endotransglucosylase/hydrolase
188 (XTH) family, were also present, with expression data shown in Fig. 5E (Supplementary Table 4 and
189 5). Additionally, the SKU similar (sks) family, coding for proteins of unknown function, and

190 gibberellin 20-oxidase 3 (GA20ox3) involved in gibberellin synthesis were included, and their
191 expressions were significantly suppressed as well (Fig. 5F and 5G, Supplementary Table 6 and 7).
192 Moreover, within the "unidimensional cell growth" category was PIN1 from the auxin transporter PIN
193 family, and the expression of many from the PIN family (PIN1/2/3/7) was also reduced (Fig. 5H,
194 Supplementary Table 8).
195

196 **Discussion**

197 The most significant finding of this study is that coumarin suppresses the expression of genes coding
198 for proteins involved in the reconfiguration of the cell wall, such as expansins, suggesting that one of
199 the final targets of coumarin's germination inhibitory effect is the cell wall. Expansins are a type of
200 plant cell wall protein that plays a crucial role in plant growth and development[15,16]. They have a
201 distinct structure comprising two domains: an N-terminal domain structurally similar to glycoside
202 hydrolase family 45 (GH45) and a C-terminal domain distantly related to group 2 grass pollen
203 allergens. While no enzymatic activity has been reported for expansins, they can induce pH-dependent
204 wall extension and stress relaxation, appearing to weaken non-covalent bonds between cell wall
205 polysaccharides. Expansins are known to play important physiological roles in various plant growth
206 and developmental processes, including cell enlargement, fruit softening, pollen tube growth, root hair
207 emergence, and abscission.

208

209 The suppression of expansin gene expression by coumarin is significant. Furthermore, coumarin also
210 downregulated the expression of genes coding for endoxyloglucan transferase A4 (XTH5) and α-
211 xylosidase1 (XYL1), which are known to interact with xyloglucans, a hemicellulose abundantly
212 present in cell walls and critical for cellular elongation growth[17,18]. The SKU similar (sks) family

213 proteins, while functionally uncharacterized, may be implicated in the biosynthesis of primary cell
214 walls in coordination with SKU[19]. Thus, similar to expansins, XTH5, and XYL1, coumarin may target
215 these proteins to inhibit cellular elongation growth.

216

217 Cell wall reorganization, directed by genes such as expansins, enables cellular elongation growth by
218 orienting cellulose fibers in a specific direction[20]. However, seeds treated with coumarin, exhibiting
219 reduced expression of such genes, would likely be hindered in this directional elongation growth. The
220 inhibition of expansin expression induction could potentially interfere with the final stages of
221 germination, where the radicle breaks through the seed coat. Notably, this suppression of expression
222 was evident at the 24-hour mark, indicating that the inhibition of cell wall reconfiguration occurs early
223 before various other changes can occur.

224

225 Our research also showed that genes involved in the gibberellin synthesis pathway were
226 downregulated by coumarin, linking cell wall reconfiguration to this suppression. Gibberellins (GA)
227 control various biological events associated with germination, including cell elongation, which
228 involves cell wall reconstruction[21,22]. There are also reports of GA inducing expansin
229 expression[23,24,25,26]. For instance, in tall fescue (*Festuca arundinacea*) leaves, GA was shown to induce

230 expansin expression differently between fast-growing and dwarf cultivars[23]. Similarly, GA likely
231 induces expansin expression during seed germination.
232
233 Studies on the relationship between plant hormones, including GA, and coumarin provide essential
234 insights into coumarin's mechanism of germination inhibition. Particularly notable is Chen's discovery
235 that coumarin reduces the synthesis of gibberellin, a hormone crucial for germination and elongation
236 growth[12]. These findings are consistent with our transcriptomic analysis. Additionally, Bruno et al.
237 demonstrated that the expression of the auxin transporter PIN family in Arabidopsis roots is suppressed
238 by the addition of coumarin[27]. Our results clarify that this effect is also present in pre-germinative
239 seeds. Therefore, it is plausible that coumarin's impact on GA and auxin plays a vital role in the
240 inhibition of germination.
241
242 Another important discovery in this research is the significant suppression by coumarin of genes
243 coding for ribosomal proteins. In line with our findings, Zhang et al., through transcriptomic analysis,
244 have also reported the inhibition of expression of these genes by coumarin in *Eleusine indica*.
245 Furthermore, Zhang et al. have reported that the synthesis of aminoacyl tRNAs is inhibited as a result
246 of their metabolomic analysis[14]. We examined gene expression at 24 hours post-coumarin treatment,

247 while Zhang et al. did so after 72 hours. Despite the difference in timing of the analyses, coumarin
248 consistently inhibits the expression of genes encoding components of ribosomes. Therefore, the
249 translation system in seeds is likely another major target of coumarin. However, the pathway through
250 which coumarin affects the synthesis of ribosomal components remains unclear. Ribosome biogenesis
251 is a process that demands a substantial energy investment from the cell, so it is conceivable that seeds
252 exposed to chemical stressors like coumarin may suppress ribosome biogenesis. Indeed, in eukaryotic
253 cells such as yeast and animal cells, ribosome biogenesis is known to be intricately regulated[28,29]. Yet,
254 it is currently unclear whether plants possess such a system. Moreover, since these suppressions of
255 expression were observed as early as 24 hours, it implies that inhibition of ribosome assembly occurs
256 at an early stage before various changes take place.

257

258 Additionally, our study has discovered that benzene rings and ester bonds are the structures responsible
259 for coumarin's effects. Knowledge regarding the relationship between coumarin's structure and activity
260 is limited and largely dates back to decades-old research by Mayer et al., which indicated the
261 importance of double bonds in dihydrocoumarins for germination inhibition[7]. Our germination tests
262 using three types of coumarin analogs suggest that benzene rings and ester bonds play key roles in its
263 effects, while double bonds are not crucial. Comparing the structures of coumarin with a double bond

264 and dihydrocoumarin without it, the change due to the absence of the double bond is minimal, limited
265 to a slight protrusion of the third carbon atom, contributing to the perceived non-essentiality of the
266 double bond. However, our results conflict with those of Mayer, who reported that the loss of the
267 double bond could attenuate germination inhibition[7]. While this discrepancy could be due to different
268 experimental conditions, such as the plant species used, the details remain unclear.

269

270 Our research also shows that the effects of the coumarin family depend on one of the lipophilicity
271 indices, TPSA. Mayer et al. have conducted extensive experiments on the effects of substituent
272 introduction to coumarin, showing that substituents in the order of NO_2, OH, and Cl may decrease
273 germination inhibition[7]. This order aligns with the TPSA values (NO_2: 49.36, OH: 20.23, Cl: 0). Given
274 that two independent studies have arrived at similar results, TPSA is likely a significant factor affecting
275 the germination inhibitory effect of the coumarin family, regardless of the experimental conditions.
276 Additionally, TPSA is known to be more closely associated with cell membrane permeability
277 compared to LogP, another lipophilicity index[30]. Therefore, the introduction of substituents to the
278 coumarin skeleton might alter germination inhibition by changing cell membrane permeability.
279 However, it is important to note that discussions regarding TPSA and cell membrane permeability
280 have primarily focused on mammalian cells.

Furthermore, the ability to quantitatively assess germination inhibition using TPSA paves the way for more efficient searches for more potent coumarin derivatives. Such derivatives hold promise for applications in weed control.

Additionally, this study suggests that the seed coat is unlikely to be the primary site of coumarin action. The seed coat serves as the first line of defense against the external environment and plays a critical role in relaying environmental conditions to the interior[9]. Furthermore, Aliotta et al. have shown that the removal of the seed coat significantly diminishes the germination inhibitory effect of coumarin[10]. On the other hand, our analyses of the seed coat's cross-sectional structure and water permeability, which have a substantial impact on germination, did not reveal any significant effects of coumarin. While there remains room for further analysis, it seems that the seed coat may not be the main site of action for coumarin. Additionally, the absorption spectrum of the seed extract suggests that coumarin penetrates the seed within 24 hours. It is highly probable that coumarin exerts a more direct effect inside the seed.

297 Considering the overall results obtained, it is possible that coumarin negatively regulates the
298 expression of cell wall-associated gene groups, including expansins, and inhibits germination by
299 obstructing the reorganization of cellulose fibers in the radicle.
300

Methods

Evaluation of the importance of coumarin substructures in germination inhibition

Coumarin (Tokyo Chemical Industry, Tokyo, Japan) was employed as a positive control. For the assessment of the benzene ring, α-pyrone (Sigma-Aldrich Japan, Tokyo, Japan) was utilized, while 3,4-dihydrocoumarin (Tokyo Chemical Industry, Tokyo, Japan) was used for the evaluation of the double bond, and β-tetralone (Sigma-Aldrich Japan, Tokyo, Japan) for the ester bond assessment. These compounds were diluted with water and prepared at a concentration of 0.10 mmol/L. Thirty seeds of white clover (*Trifolium repens*) were sown onto each cotton-lined petri dish, and these solutions were then added. Ion-exchanged water, without the addition of compounds, served as the negative control. Subsequently, the petri dishes were incubated in darkness at 20°C for 48 hours in an incubator (A4201, IKUTA Sangyo Co, Ltd, Japan). The number of germinations per petri dish was counted, and the percentage of germinated seeds out of the total number sown was calculated as the germination rate. The experiment to investigate the effect of one type of compound was repeated ten times, using a total of 300 seeds.

Correlation analysis between cheminformatics-estimated indices and germination inhibition effects

318 As members of the coumarin family, six compounds were prepared: coumarin (KANTO CHEMICAL
319 CO., INC., Tokyo, Japan), 3,4-dihydrocoumarin, 7-methoxy coumarin (Tokyo Chemical Industry,
320 Tokyo, Japan), aesculetin (Apollo Scientific Ltd, Manchester, UK), psoralen (Tokyo Chemical
321 Industry, Tokyo, Japan), and umbelliferone (FUJIFILM Wako Pure Chemical Co., Osaka, Japan).
322 These compounds were diluted with water and prepared at a concentration of 0.10 mmol/L, and the
323 germination rates were calculated in a manner similar to the above. Various indices estimated by
324 cheminformatics for each compound were calculated using the following methods. The Topological
325 Polar Surface Area (TPSA), and the octanol/water partition coefficient (LogP) were computed using
326 SwissADME (http://www.swissadme.ch/)[31]. The molecular surface area and van der Waals volume
327 were determined using winmostar (V11.5.8, https://winmostar.com/en/). The correlations between
328 these indices and the germination rates were investigated.

329

330 **Observation of seed cross-sections with scanning electron microscopy**

331 Pea (*Pisum sativum*) seeds were selected for their ease of observation. Three seeds were immersed for
332 24 hours in either water or a coumarin solution adjusted to 10 mmol/L within an incubator set to 20°C
333 under dark conditions. Subsequently, the seeds were spread on cotton-lined Petri dishes, and an
334 additional 10 mL of the same solution was added. After another 24 hours, the seeds were allowed to

air-dry naturally for 24 hours. The fractured cross-sections of these seeds were then prepared using a razor blade (FEATHER Safety Razor Co., Ltd) and sputter-coated with Pt-Pd. The coated seeds were observed under a scanning electron microscope (SEM; S-3400N, Hitachi, Ltd, Osaka, Japan) at an accelerating voltage of 15 kV and a magnification of 250x. Images of the seeds were captured from five locations on the cross-sections of the three cleaved seeds. The thickness from the palisade tissue to the spongy parenchyma of the seeds was measured using ImageJ Fiji (ver. 1.54f, https://imagej.nih.gov)[32]. Additionally, the thickness of each tissue was measured in the same manner.

Measurement of seed water absorption

Ten pea seeds were permeated in either water or a coumarin solution prepared at 10 mmol/L, and their surrounding moisture was removed with a paper towel before the fresh weight was measured over time. Fresh weight measurements were taken hourly from 1 to 24 hours. The seeds were then dried for over 24 hours in a constant temperature drying oven set to 90°C, and the dry weight was measured. The water absorption of the seeds from 1 to 24 hours was calculated based on the difference between each time point's fresh weight and the dry weight.

351 **Detection of coumarin within seed interiors**

352 Five pea seeds were immersed in a coumarin solution prepared at 10 mmol/L and were incubated for
353 24 hours in an incubator set at 20°C. Subsequently, the seed coats were delicately scored with a razor
354 blade and removed, after which the inner part of the seed coat was pulverized using a mortar and pestle.
355 The absorption spectrum of 1 mL of this pulverized solution, diluted 100-fold, was measured using a
356 spectrophotometer (PD-3000 Uve, APEL Co., Ltd., Saitama, Japan).

357

358 **Transcriptome analysis by RNA-Seq**

359 Arabidopsis seeds, known for their well-characterized genetic information, were used. Initially, 20 mg
360 of seeds were treated with either a 10 mmol/L coumarin solution or water and allowed to incubate for
361 24 hours. Three biological replicates were used for each condition. The seeds were then manually
362 crushed with a pestle, and RNA extraction was performed using ISOSPIN Plant RNA (NIPPON GENE,
363 Japan). Subsequently, RNA concentration was measured using the Qubit RNA BR Assay Kit (Thermo
364 Fisher Scientific, CA, USA). RNA samples were sent to Novogene (Singapore) for RNA sample
365 quality testing using a Bioanalyzer (Agilent, California, USA), library construction using the
366 NEBNext Ultra II RNA Library Prep Kit for Illumina, and sequencing on the NovaSeq 6000 platform
367 (Illumina, California, USA). The resulting FASTQ data were analyzed using RaNA-seq

368 (https://ranaseq.eu/)[33], where differential gene expression analysis was conducted based on the
369 DESeq2 method[34]. In addition, GSEA[35,36] was performed on these DEGs. The GSEA, including
370 pathway and gene ontology analyses, determined the extent of impact by the normalized enrichment
371 score (NES). The pathway analysis referenced the KEGG PATHWAY Database, and the gene ontology
372 analysis was carried out with reference to the database of the National Center for Biotechnology
373 Information (NIH), analyzing biological process, cellular component, and molecular function domains.

374

375 **Utilization of generative AI**

376 ChatGPT (https://chat.openai.com/) was employed to rewrite the manuscript into more appropriate
377 English and to proofread the text. The correctness of the revisions was subsequently verified by the
378 authors.

379

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464

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Author Contributions

K.F., T.O., S.H., M.Y., and K.M. conceptualized the research plan. S.H., T.O., M.Y., H.M., M.K., and C.E. collected the germination test data. S.H. and T.O. conducted the cheminformatics analysis. M.Y., M.K., K.F., M.N., and N.R. carried out the SEM observations. K.F. performed the measurements of water absorption. R.I. and K.T. were engaged in the experiments to investigate the spectral properties of seed extractions. K.F., S.H., T.O., T.Y., and N.R. executed RNA extraction. K.F., S.H., T.O., N.R., Y.N., and K.M. conducted the bioinformatics analysis. K.F. and R.I. completed the statistical analysis.

As Ph.D. holders, N.R. and Y.N. provided advisory input to the research. The manuscript was drafted by K.F., S.H., and T.O., edited by Y.N. The manuscript was finalized by K.F., S.H., T.O., M.Y., N.R., Y.N., and K.M. The manuscript was reviewed by all authors.

Data Availability Statement

The raw sequencing data for each sample have been deposited in the DDBJ Sequence Read Archive (https://www.ddbj.nig.ac.jp/dra/index-e.html; accession numbers DRR546110–DRR546115).

Competing Interests

The authors declare no competing interests.

493 **Figure Legends**

494 **Figure 1.** Loss of the benzene ring and ester linkage from coumarin attenuates its germination
495 inhibitory effect. (**A–D**) Chemical structures of coumarin-like substances used in this study are shown
496 (coumarin (**A**),α-pyrone (**B**), β-tetralone (**C**), and 3,4-dihydrocoumarin (**D**)). (**E**) Germination rates of
497 white clover *(Trifolium repens)* seeds after 48 hours of incubation with the addition of coumarin-like
498 substances (n = 300). The solutions used in the experiments were prepared at a concentration of 0.10
499 mmol/L. Below the graph, the presence or absence of the benzene ring, ester bond, and double bond
500 within the lactone ring for each substance is indicated (+ means the structure is present, - means it is
501 absent). Group comparisons of germination rates were made using Chi-squared tests, where the
502 asterisk (*) indicates statistical significance ($p < 0.05$). Error bars represent the 95% confidence
503 interval of the proportion.

504

505 **Figure 2.** A strong correlation is observed between TPSA and the germination inhibition effect. (**A–**
506 **D**) Structures of additional compounds used in this study, in addition to coumarin and
507 dihydrocoumarin, as shown in Fig. 1 (7-methoxy-coumarin (**A**), aesculetin (**B**), psoralen (**C**), and
508 umbelliferone (**D**)). (**E–H**)Relationships between germination rates of seeds treated with coumarin
509 derivatives and four calculated structural indicators are depicted. The y-axis shows the germination

510 rates of seeds treated with each member of the coumarin family (n = 300), and the x-axis shows the
511 calculated indicators from the structure (Molecular Volume (**E**), Molecular Surface Area (**F**), LogP
512 (**G**), and TPSA (**H**)). Pearson's correlation coefficient r and the p-value from the test of no correlation
513 are provided. For TPSA, where a significant correlation was observed, a scatter plot with a least-
514 squares linear regression model is shown.

515

516 **Figure 3.** No observable changes in seed coat structure with coumarin treatment. (**A**) SEM image of
517 a pea (*Pisum sativum*) seed coat cross-section after 72 hours of treatment with coumarin. Seeds were
518 sputter-coated with Pt-Pd, observed under an acceleration voltage of 1.5 kV and magnification of 250x.
519 PAL indicates palisade parenchyma, HOU represents hourglass cells, and COM signifies compressed
520 parenchyma. Scale bar = 100 μm. (**B**) Thickness measurements of pea seed coat tissues treated with
521 coumarin. Measurements were obtained from images captured using the same method as Fig. 3A and
522 analyzed with ImageJ Fiji software. "All" indicates the total thickness from palisade parenchyma to
523 compressed parenchyma. The thickness of each tissue was compared between coumarin-treated and
524 untreated seeds using Student's t-test. n.s. denotes no statistical significance ($p > 0.05$), and error bars
525 represent the 95% confidence interval for the mean. (**C**) Water absorption of pea seeds from 1 to 24
526 hours post-immersion. Absorption was calculated based on the difference between wet weight

527 measured post-absorption and dry weight after drying. No significant differences were observed at any
528 time point ($p > 0.05$). (**D**) Absorption spectra of homogenates from ten pea seeds immersed in a
529 coumarin solution for 24 hours. Experiments were conducted after removing seed coats using a 10
530 mol/L coumarin solution, replicated three times. Coumarin's maximum absorption wavelength at 270
531 nm is indicated with a dashed line. Absorbance at 270 nm was compared between treatments with and
532 without coumarin using Student's t-test, with the asterisk (*) indicating statistical significance ($p <$
533 0.05).

534

535 **Figure 4.** Down-regulation in genes related to ribosomes and expansins by coumarin. (**A–B**) The
536 heatmaps display the expression ratio of RPL family (**A**) and RPS family genes (**B**) between coumarin-
537 treated and control groups obtained through RNA-Seq. Experiments were replicated thrice for each
538 group, with columns corresponding to the expression profiles of individual samples. (**C**) The bar plot
539 represents the log2-transformed expression ratios of expansin family genes between the coumarin-
540 treated and control groups. The asterisk (*) denotes statistical significance based on the adjusted p-
541 value ($padj<0.05$).

542

543 **Figure 5.** Coumarin Addition Impacts Multiple Pathways. (**A**) Rug plots and enrichment score walk

544 from GSEA based on the KEGG Pathways database, showing the maximum distance from zero along

545 the walk, calculated as the enrichment score. This analysis reveals the impact of coumarin on eight

546 pathways in Arabidopsis, with particularly interesting data for four pathways shown. (**B–D**) Bar plots

547 display the normalized enrichment score (NES) from GSEA using the NIH Gene Ontology database.

548 The top five activated and repressed pathways are shown for each gene ontology category (biological

549 process (**B**), cellular component (**C**), and molecular function (**D**)). (**E–H**)Bar plots show the log2

550 expression ratio between the coumarin-treated and control groups for genes of the XYL1 and XTH

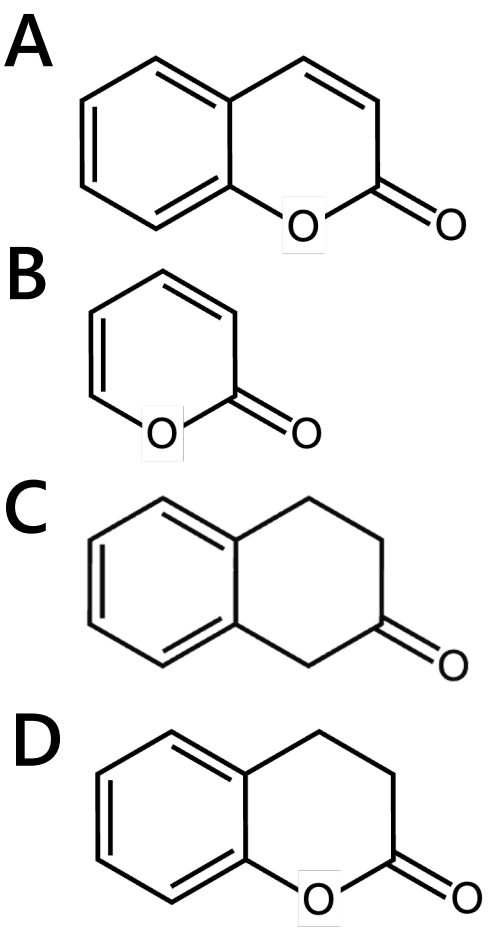
551 families (**E**), the sks family (**F**), GA20ox1/3 genes (**G**), and the PIN family genes (**H**). The asterisk

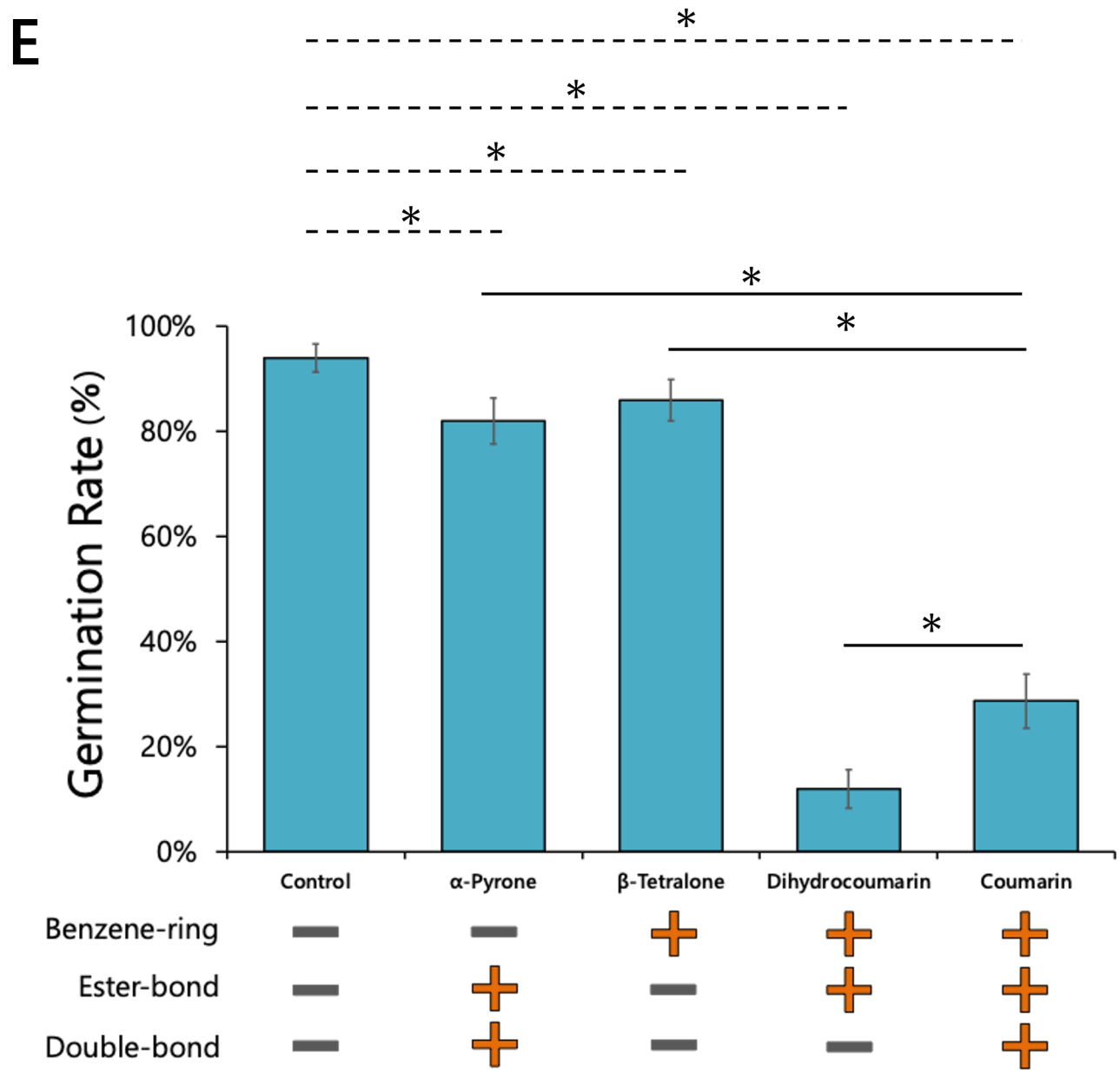
552 (*) indicates statistical significance based on the adjusted p-value (padj < 0.05).

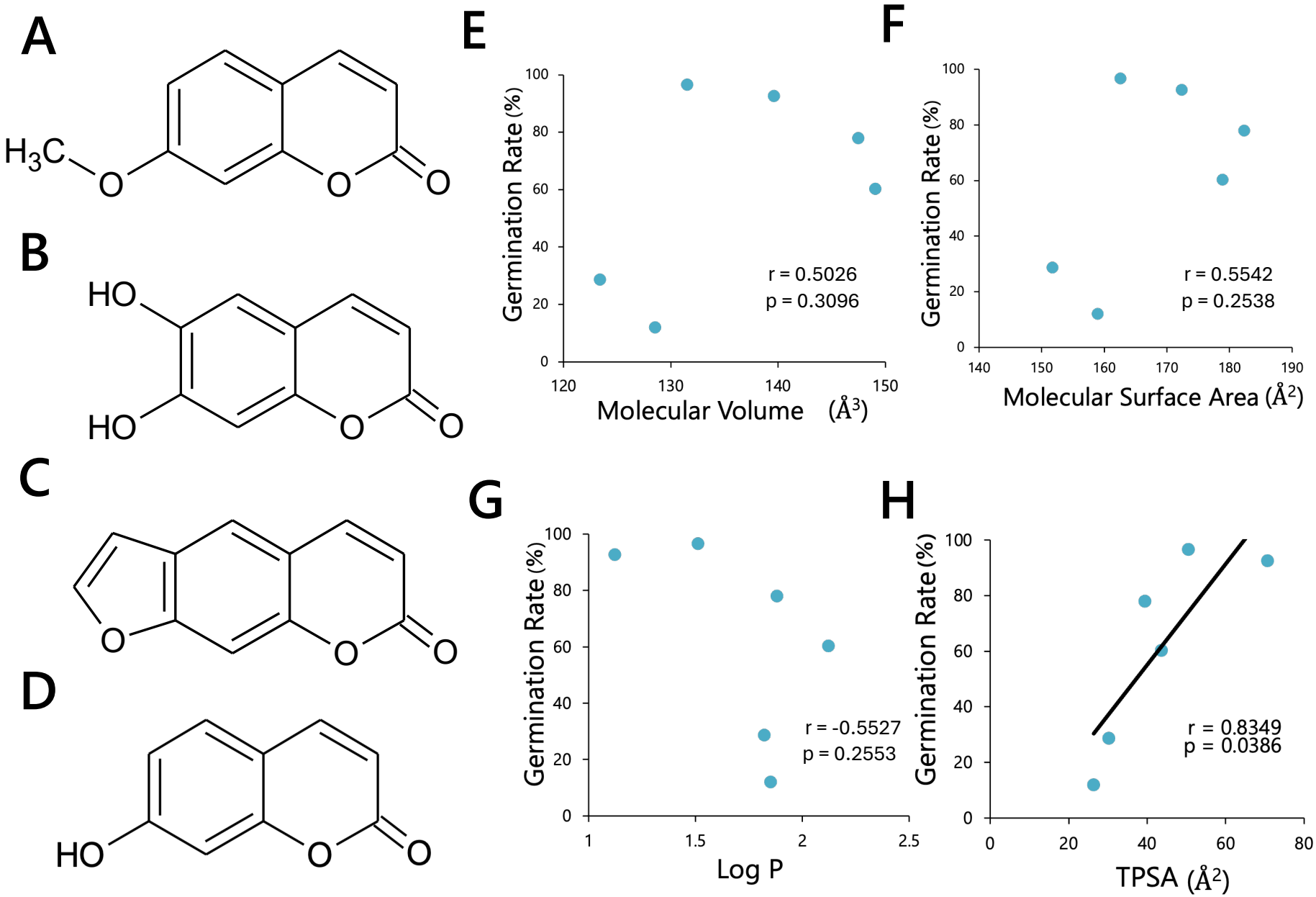
553

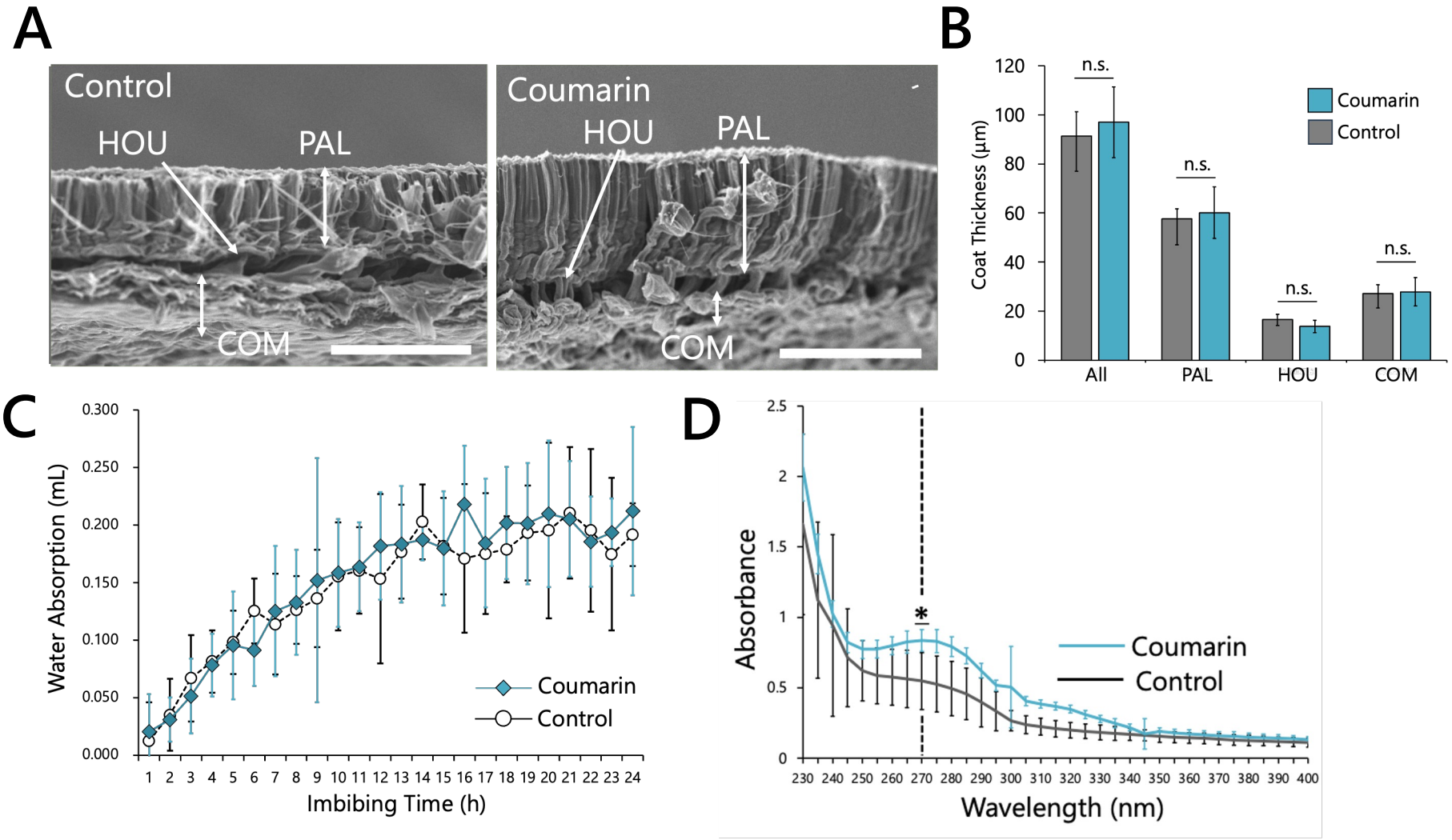
Table 1. GSEA Pathway Analysis Results

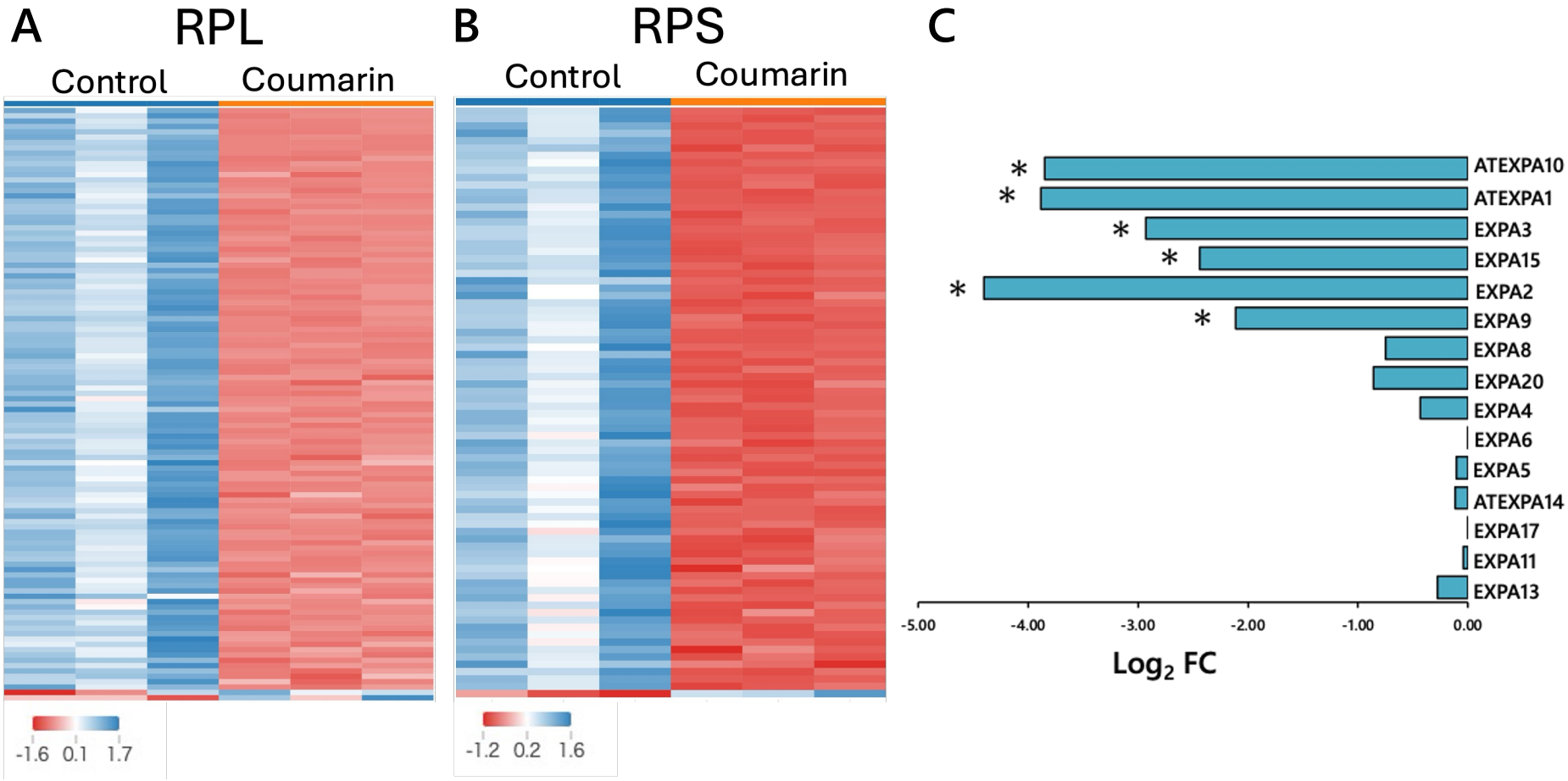
Pathway_ID	Pathway	NES	padj
ath03010	Ribosome	-2.409	0.011
ath_M00177	Ribosome, eukaryotes	-2.290	0.011
ath_M00049	Adenine ribonucleotide biosynthesis, IMP => ADP,ATP	-1.812	0.011
ath_M00087	beta-Oxidation	1.969	0.011
ath00480	Glutathione metabolism	2.004	0.013
ath_M00113	Jasmonic acid biosynthesis	2.053	0.018
ath00945	Stilbenoid, diarylheptanoid and gingerol biosynthesis	1.902	0.024
ath00230	Purine metabolism	-1.674	0.049

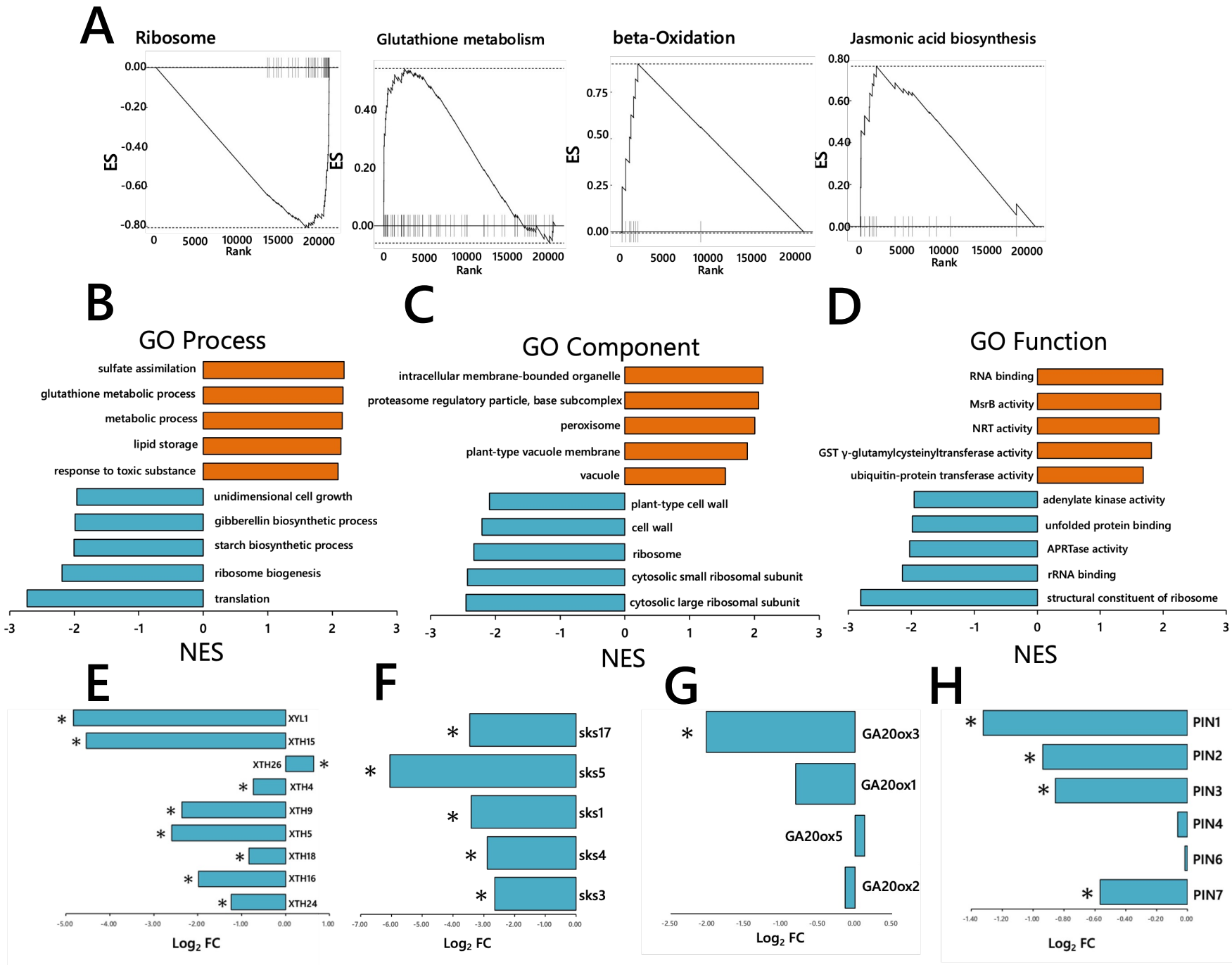

A
B
C
D
O
O


E
*
*
*
*
*
*
*
Germination Rate (%)
100%
80%
60%
40%
20%
0%
Control
α-Pyrone
β-Tetralone
Dihydrocoumarin
Coumarin
Benzene-ring
Ester-bond
Double-bond


A
H_3C
O
O
O
B
HO
HO
O
O
C
O
O
O
D
HO
O
O
E
Germination Rate (%)
Molecular Volume (Å³)
r = 0.5026
p = 0.3096
F
Germination Rate (%)
Molecular Surface Area (Å²)
r = 0.5542
p = 0.2538
G
Germination Rate (%)
Log P
r = -0.5527
p = 0.2553
H
Germination Rate (%)
TPSA (Å²)
r = 0.8349
p = 0.0386


A
Control
HOU
PAL
COM
Coumarin
HOU
PAL
COM
B
Coat Thickness (µm)
120
100
80
60
40
20
0
n.s.
n.s.
n.s.
n.s.
Coumarin
Control
All
PAL
HOU
COM
C
Water Absorption (mL)
0.300
0.250
0.200
0.150
0.100
0.050
0.000
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
Imbibing Time (h)
Coumarin
Control
D
Absorbance
2.5
2
1.5
1
0.5
0
*
230 240 250 260 270 280 290 300 310 320 330 340 350 360 370 380 390 400
Wavelength (nm)
Coumarin
Control


A
RPL
Control
Coumarin
-1.6 0.1 1.7
B
RPS
Control
Coumarin
-1.2 0.2 1.6
C
ATEXPA10
ATEXPA1
EXPA3
EXPA15
EXPA2
EXPA9
EXPA8
EXPA20
EXPA4
EXPA6
EXPA5
ATEXPA14
EXPA17
EXPA11
EXPA13
-5.00
-4.00
-3.00
-2.00
-1.00
0.00
Log2 FC


A
Ribosome
Glutathione metabolism
beta-Oxidation
Jasmonic acid biosynthesis
ES
Rank
B
GO Process
sulfate assimilation
glutathione metabolic process
metabolic process
lipid storage
response to toxic substance
unidimensional cell growth
gibberellin biosynthetic process
starch biosynthetic process
ribosome biogenesis
translation
NES
C
GO Component
intracellular membrane-bounded organelle
proteasome regulatory particle, base subcomplex
peroxisome
plant-type vacuole membrane
vacuole
plant-type cell wall
cell wall
ribosome
cytosolic small ribosomal subunit
cytosolic large ribosomal subunit
NES
D
GO Function
RNA binding
MsrB activity
NRT activity
GST γ-glutamylcysteinyltransferase activity
ubiquitin-protein transferase activity
adenylate kinase activity
unfolded protein binding
APRTase activity
rRNA binding
structural constituent of ribosome
NES
E
XYL1
XTH15
XTH26
XTH4
XTH9
XTH5
XTH18
XTH16
XTH24
Log2 FC
F
sks17
sks5
sks1
sks4
sks3
Log2 FC
G
GA20ox3
GA20ox1
GA20ox5
GA20ox2
Log2 FC
H
PIN1
PIN2
PIN3
PIN4
PIN6
PIN7
Log2 FC