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1 The rice G protein γ subunit *qPE9-1* positively regulates grain-filling

2 process by interacting with abscisic acid and auxin

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24 ## Abstract

25 The rice genome contains a single G_{α} (*RGA1*) and G_{β} (*RGB1*) and five G_{γ} subunits. Recent
26 genetic studies have shown that *DEP1/qPE9-1*, an atypical putative G_{γ} protein, is responsible
27 for dense and erect panicles, but the biochemical and molecular mechanisms underlying
28 control of grain size are not well understood. Here, we report that plants carrying *qPE9-1*
29 have more endosperm cells per grain than plants contain the *qpe9-1* allele. The *qPE9-1* line
30 has a higher rate and longer period of starch accumulation than the *qpe9-1* line. Additionally,
31 the expression of several key genes encoding enzymes catalyzing sucrose metabolism and
32 starch biosynthesis is higher in the *qPE9-1* line than in the *qpe9-1* line, especially from the
33 mid to late grain-filling stage. Grains of the *qPE9-1* line also have higher contents of two
34 phytohormones, ABA and IAA. Exogenous application of ABA or IAA enhanced starch
35 accumulation and the expression of genes encoding grain-filling-related enzymes in the
36 grains of *qPE9-1*, whereas only IAA produced these effects in *qpe9-1*. Based on these results,
37 we conclude that *qPE9-1* promotes endosperm cell proliferation and positively regulates
38 starch accumulation largely through ABA and IAA, which enhance the expression of genes
39 encoding starch biosynthesis during the late grain-filling stage.

40

41 **Keywords:** G-protein, *qPE9-1*, starch biosynthesis, grain filling, hormones, *Oryza sativa*

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47 ## Introduction

48 Rice is the major staple food for more than half of the global population. This crop provides
49 approximately 20% of the global dietary energy supply and plays vital roles in sustaining
50 global food security. Understandably, improving rice productivity is the predominant target
51 in rice breeding because of the continuing increase in the global population. Rice yield is a
52 complex trait that is influenced by numerous components, including the number of panicles
53 per plant, the number of grains per panicle and the grain size (i.e., 1000-grain weight).
54 Furthermore, these components interact with each other in most cases. Typically, the rice
55 grain yield is determined by both the grain capacity (i.e., the grain number and size) and the
56 grain-filling efficiency; the former is controlled by the numbers of spikelets per panicle and
57 the numbers and sizes of endosperm cells per spikelet/grain, both of which determine the
58 final grain number per panicle and the grain size. The grain-filling efficiency is closely
59 related to the grain-filling rate and duration.

60 The heterotrimeric G protein (hereafter G protein)-mediated signal transduction pathway is
61 considered one of the most important signaling mechanisms and regulates various important
62 physiological and molecular processes in both mammals and higher plants (Urano *et al.*,
63 2014). In this signaling pathway, the G protein, which is well-known to consist of three
64 different subunits (α, β and γ) acts as a signal intermediator in the transduction of numerous
65 external signals (Milligan & Kostenis, 2006). Arabidopsis G-protein Gamma subunit 3
66 (*AGG3*), which represents a novel class of canonical γ subunits in Arabidopsis, is widely
67 spread throughout the plant kingdom but is not present in animals (Urano *et al.*, 2013).
68 Recently, *AGG3* has been proposed to be an important regulator of organ size and a mediator
69 of stress responses in *Arabidopsis*. Roy Choudhury *et al*. (2013) overexpressed *AGG3* in
70 *Camelina* and found that it increased the seed size, seed mass and seed number per plant by
71 15%-40%, effectively resulting in a significantly higher oil yield per plant. In addition,

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72 recently *AGG3* has been shown to affect the guard cell K^+ channel, morphological
73 development, ABA responses and cell proliferation (Chakravorty *et al.*, 2011; Li *et al.*, 2012;
74 Roy Choudhury *et al.*, 2013). These observations draw a strong link between the roles of
75 *AGG3* in regulating two critical yield parameters (seed traits and plant stress responses) and
76 reveal an effective biotechnological tool to dramatically increase agricultural crop yield.

77 Homologs of *AGG3* in rice have been identified as important quantitative trait loci (QTL)
78 for grain size and yield. The major rice QTL [i.e., *GRAIN SIZE 3* (*GS3*) and *DENSE AND*
79 *ERECT PANICLE1* (*DEP1*)/*PANICLE ERECTNESS* (*qPE9-1*)] have 29.4% and 22.5%
80 amino acid sequence identities, respectively, with *AGG3* (Li *et al.*, 2012). As an atypical G_γ
81 protein, rice *GS3* has been proposed to negatively affect cell proliferation, whereas
82 *DEP1/qPE9-1* plays a positive role (Fan *et al.*, 2006; Huang *et al.*, 2009). These findings
83 suggest that *AGG3* and its homologs in rice may have divergent functions.

84 In the present study, we show that *qPE9-1* stimulates endosperm cell proliferation, grain
85 filling and consequently the final grain weight. The results also indicate that *qPE9-1* exerts its
86 effects at least in part by controlling abscisic acid (ABA) and indole-3-acetic acid (IAA)
87 biosynthesis.

88

89 ## Materials and Methods

90 ### Plant material and treatments

91 The experiment was carried out at the farm of Yangzhou University (32°30’N, 119°25’E)
92 during the rice (*Oryza sativa*) growing season (from early May to early September).
93 Transgenic rice *qPE9-1* and the donor Wuyunjing 8 (*qpe9-1*) were grown in the field (see
94 Zhou *et al.*, 2009). At the heading stage, 500 uniformly growing and headed panicles (1-2
95 panicles per plant) were chosen, and spikelets on the selected panicles with the same

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96 flowering date were labeled for each cultivar/line. The flowering date and position of each
97 spikelet on the labeled panicles were recorded. Approximately 45 labeled panicles were
98 sampled at each time point from flowering to maturity. Half of the sampled grains were
99 frozen in liquid nitrogen for at least 2 min and then stored at -80°C for subsequent analyses.
100 The other half of the grains were dried at 80°C for approximately 72 h to a constant weight
101 and used for the starch analyses.

102 The hormone treatment consisted of 80 plastic pots with planted rice (three hills per pot)
103 maintained under open field conditions. Each pot (0.6 m in height with 0.5 m and 0.3 m top
104 and bottom diameters, respectively) was filled with sandy loam soil that contained the same
105 nutrient contents as the field soil. The sowing date and cultivation were the same as those for
106 the field experiment. After flowering, either 50 μM ABA or 110 μM IAA was sprayed at a
107 rate of 10 ml per pot on the top of the plants (spikes) every 3 days. Both ABA and IAA were
108 applied between 16:00 h and 18:00 h. All of the solutions contained ethanol and Tween 20 at
109 final concentrations of 0.1% (v/v) and 0.01% (v/v), respectively. The control plants were
110 sprayed with the same volume of deionized water containing the same ethanol and Tween 20
111 concentrations. Each treatment consisted of 13 pots, and the labeled spikelets were sampled.

112

113 **Isolation and counting of endosperm cells**

114 The endosperm cells of the grains were isolated and counted according to the procedures
115 described by Singh & Jenner (1982). Briefly, fixed grains (10 grains from five labeled
116 panicles) were transferred to 0.7:1 (v:v) ethanol:water and dehulled. The dehulled grains
117 were transferred to 0.5:1 (v:v) ethanol, 0.25:1 (v:v) ethanol and finally to distilled water for
118 5~7 h prior to dissection of the endosperms. The endosperms were isolated under a dissecting
119 microscope and dyed using Delafield's hematoxylin solution for 24~30 h, washed several

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120 times with distilled water and then hydrolyzed in a 0.1% cellulase solution at 40°C. The
121 degree of recovery of the cells after digestion was 80~95%. The isolated endosperm cells
122 were diluted to 2~10 ml according to the developmental stage of the endosperm, from which
123 5 subsamples (20 ml per subsample) were transferred to a counting chamber (1-cm^2 area).
124 The endosperm cell number in 10 grids per counting chamber was counted using a light
125 microscope. The number of nuclei was counted as the number of endosperm cells. The total
126 endosperm cell numbers were calculated using the following equation (Liang *et al.*, 2001):

$$\text{Endosperm cell number} = \frac{\dfrac{\frac{\text{cell number}}{\text{grid}} \times \text{counting chamber area}}{\text{area of each grid}} \times \text{total volume of sample}}{\text{volume of each subsample}}$$

127

128 **Gene expression analysis**

129 Total RNA was extracted from the grains (10~20 grains from five labeled panicles) using the
130 RNAprep Pure Plant Kit (cat. no. DP441; Tiangen, Beijing, China). The HiScript II Q Select
131 RT SuperMix (Vazyme, Nanjing, China) was used for cDNA synthesis. The transcript level
132 of each gene was measured by qRT-PCR using the 7500 Real-Time PCR System (ABI) with
133 the PowerUp™ SYBR® Green Master Mix (Thermo Fisher Scientific, San Jose, USA). Gene
134 expression was quantified during the logarithmic phase using expression of the housekeeping
135 gene *Ubq* (LOC_Os03g13170) as an internal control. Three biological replicates were
136 performed for each experiment. The primer sequences used for qRT-PCR are described by
137 Wang *et al.* (2013).

138

139 **Hormone quantification**

140 The IAA and ABA levels were determined by Zoonbio Biotechnology Co., Ltd (Nanjing,
141 China). Approximately 0.5 g of the samples were ground in a precooled mortar that contained

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142 5 ml of extraction buffer composed of isopropanol/hydrochloric acid. The extract was shaken
143 at 4°C for 30 min. Then, 10 ml of dichloromethane was added, and the sample was shaken at
144 4°C for 30 min and centrifuged at 13,000 rpm for 5 min at the same temperature. We
145 extracted the lower organic phase. The organic phase was dried under N_2, dissolved in 150 µl
146 of methanol (0.1% methane acid) and filtered with a 0.22-µm filter membrane. The purified
147 product was subjected to high-performance liquid chromatography-tandem mass
148 spectrometry (HPLC-MS/MS) analysis. The HPLC analysis was performed using a
149 ZORBAX SB-C18 (Agilent Technologies) column (2.1 mm × 150 mm; 3.5 mm). The mobile
150 phase A solvent consisted of methanol/0.1% methanoic acid, and the mobile phase B solvent
151 consisted of ultrapure water/0.1% methanoic acid. The injection volume was 2 µl. The MS
152 conditions were as follows: the spray voltage was 4500 V; the pressure of the air curtain,
153 nebulizer and aux gas were 15, 65 and 70 psi, respectively; and the atomizing temperature
154 was 400°C.

155

156 **Enzyme activity assays**

157 The dehulled grains (10 grains from five labeled panicles) were homogenized with a
158 prechilled mortar and pestle in 100 mM HEPES buffer (pH 7.5) containing 8 mM $MgCl_2$, 2
159 mM EDTA, 50 mM 2-mercaptoethanol, 12% (v/v) glycerol and 1% (w/v)
160 polyvinylpyrrolidone (PVP). After centrifugation at 30,000 x g for 10 min at 4°C, the
161 supernatant was desalted through a dialytic membrane. The dialysis buffer contained 5 mM
162 HEPES-NaOH, pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA and 0.5 mM DTT. The enzyme activity
163 of SUS (in the cleavage direction), SS and BE was determined as described by Nakamura *et*
164 *al.* (1989), Jiang *et al.* (2003) and Tang *et al.* (2009). The grain starch contents were
165 determined according to Lü *et al.* (2008).

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166

167 **Protein blotting analysis**

168 Rice grains (~10 grains from five labeled panicles of different plants) were homogenized in
169 TEDM buffer (20 mM Tris/HCl, pH 7.5, 1 mM DTT, 5 mM EDTA and 10 mM $MgCl_2$)
170 containing a complete protease inhibitor cocktail (Roche). The homogenate was centrifuged
171 at 6,000 x g for 30 min at 4°C to remove cellular debris, and the supernatant was clarified by
172 centrifugation at 5,000 x g for 90 min at 4°C. The soluble proteins were separated by SDS–
173 PAGE on a 10% gel and blotted onto a Polyvinylidene difluoride (PVDF) membrane
174 (Millipore, Bedford, MA, USA). The DEP1/qPE9-1 (LOC_Os09g26999) and RGB1
175 (LOC_Os03g46650) antibodies were prepared by ABclonal (Wuhan, China). Serum was
176 collected from a rabbit after multiple injections of the RGB1 (full-length) protein and a
177 polypeptide (MEAPRPKSPPRYPDLC) of qPE9-1 as the antigen. The evaluation of antibody
178 valence is shown in Figure S1.

179

180 **Statistical analysis**

181 The data are presented as the mean ± SD. The SPSS 16.0 software was used for all statistical
182 analyses. Statistical significance was determined for independent biological samples using
183 Student's t-test for comparison of two groups and one-way ANOVA for comparison of three
184 or more groups. Differences were considered statistically significant when $P < 0.05$. An
185 asterisk (*) is presented when *P* □ 0.05.

186

187 **Results**

188 ***qPE9-1* positively controls grain size**

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189 Using QTL analysis, two independent research groups originally identified rice *qPE9-1* as
190 controlling the panicle morphology and grain number per panicle (Huang *et al.*, 2009; Zhou
191 *et al.*, 2009). However, the biochemical and molecular mechanisms underlying control of the
192 rice grain size and weight are largely unknown. In the present study, we compared the
193 differences in grain size of Wuyunjing 8, which contains the *qpe9-1* allele (hereafter *qpe9-1*),
194 and the *qPE9-1* transgenic line (hereafter *qPE9-1*). The results showed that the *qpe9-1* grain
195 was approximately 12% shorter than that of *qPE9-1* (Fig. 1a and f) and that *qPE9-1* had a
196 larger grain size during the grain-filling stage than *qpe9-1* (Fig. 1c). However, the width and
197 thickness of the *qpe9-1* and *qPE9-1* grains did not differ significantly (Fig. 1b and f). The
198 1000-grain weight of *qPE9-1* was remarkable higher than that of *qpe9-1* (Fig. 1g). The
199 mRNAs of *qPE9-1* are still present in *qpe9-1*, although neither the full-length protein (qPE9-
200 1, predicted molecular weight 45.2 kDa) nor a truncated protein (qpe9-1, predicted molecular
201 weight 21.5 kDa) was detected by immunoblotting with the anti-qPE9-1 antibody (Figs. 1d
202 and e). These results indicate that the full-length qPE9-1 protein promotes the grain length in
203 rice.

204

205 ***qPE9-1* endosperm cell proliferation**

206 Organ size and shape are determined by cell proliferation and expansion (Orozco-Arroyo *et*
207 *al.*, 2015). We compared the numbers of endosperm cells in cross-sections of mature grains
208 from *qpe9-1* and *qPE9-1* (Fig. 2a). No significant difference in the endosperm cell area was
209 observed between the *qpe9-1* and *qPE9-1* lines (Fig. 2b). We also found that the number of
210 endosperm cells per grain increased rapidly during early grain filling and that the peak values
211 were reached nine days after flowering (9 DAF). The number of endosperm cells per grain of
212 *qPE9-1* was significantly higher than that of *qpe9-1* (Fig. 2c). The long and short axes of the
213 *qpe9-1* endosperm cells were slightly shorter than those of *qPE9-1*, but the differences were

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214 not significant (Fig. 2d). There findings suggest that *qPE9-1* has no obvious effect on cell
215 proliferation during grain growth and development and that the increase in endosperm (grain)
216 size in *qPE9-1* results mainly from an increase in cell numbers during the early stage of grain
217 development.

218 ***qPE9-1* enhances starch biosynthesis in rice grains**

219 The starch content of the grains increased rapidly after flowering in both genotypes. However,
220 the grain of the *qPE9-1* line contained a much higher starch content than that of the *qpe9-1*
221 line, and the final starch content measured in the *qPE9-1* grain was approximately 25%
222 higher than that in *qpe9-1* (Fig. 3a). Interestingly, the maximum starch content was measured
223 at 27 DAF in *qpe9-1* and remained relatively stable until the end of grain filling, whereas the
224 starch content in *qPE9-1* increased continuously until 33 DAF (Fig. 3a). The rate of starch
225 accumulation increased rapidly after flowering, reached its maximal level at 15 DAF and
226 then decreased as the grain-filling process continued. Again, the rate of starch accumulation
227 during grain filling was almost always higher in *qPE9-1* than in *qpe9-1,* especially from the
228 mid to late grain-filling stage (from 18 to 33 DAF, Fig. 3b). These results clearly indicate that
229 *qPE9-1* positively controls the starch accumulation process during grain filling and prolongs
230 the duration of the grain-filling process. Furthermore, the total starch content in *qPE9-1* flour
231 was significantly higher than that of *qpe9-1*, suggesting that *qPE9-1* increased conversion of
232 sucrose to starch.

233

234 ***qPE9-1* stimulates the expression of genes encoding grain-filling-related enzymes**

235 Sucrose synthase (SUS), invertase (INV), ADP-glucose pyrophosphorylase (AGP), starch
236 synthases (SS and GBSS), branching enzyme (BE) and debranching enzyme (DBE) are well-
237 known to play key roles in the regulation of sucrose degradation, ADP-glucose and starch

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238 biosynthesis (Liang *et al.*, 2001; Lü *et al.*, 2008; Tang *et al.*, 2009; also see reviews by
239 Tetlow *et al.*, 2004; Keeling & Myers, 2010; Zeeman *et al.*, 2010). In this study, we found
240 that the *OsSUS3*, *OsSSIIa* and *OsBEIIb* transcript levels were significantly higher in the
241 *qPE9-1* grains than in the *qpe9-1* grains. Then, we examined the expression of these genes
242 during the entire grain-filling stage. The results showed that the transcript levels of these
243 genes were significantly higher in the *qPE9-1* grains than in the *qpe9-1* grains, especially at
244 the later grain-filling stage (Fig. 4a). These results suggest that the molecular mechanisms
245 that regulate sucrose degradation and starch biosynthesis may differ between *qpe9-1* and
246 *qPE9-1*, which may explain the differences in the final grain weight between the two
247 genotypes.

248 To elucidate the roles of these genes at the biochemical level, the activities of several
249 enzymes encoded by these genes were measured in the grains of the two genotypes during
250 grain filling. As shown in Fig. 4b, significant differences in SUS, SS, GBSS and BE activity
251 were observed between *qpe9-1* and *qPE9-1*; the dynamic changes in the activities of these
252 enzymes during grain filling were very similar to the expression patterns of the encoded
253 genes. Taken together, our results suggest that *qPE9-1* plays key roles in controlling grain
254 filling through its effects on gene expression and the activities of the encoded enzymes
255 related to sucrose metabolism and starch biosynthesis during grain filling.

256

257 ***qPE9-1* enhances the accumulation of ABA and IAA in the endosperm during grain**
258 **filling**

259 Rice grain filling is a complicated process that involves the interconversion of many
260 metabolites and the metabolism of bioactive compounds, including plant hormones. The rice
261 grain-filling process is highly regulated by plant hormones (Tang *et al.*, 2009; Zhu *et al.*,

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262 2011; Zhang *et al.*, 2016). We were interested in investigating whether *qPE9-1* controlled the
263 grain-filling process through its effects on plant hormone homeostasis. Therefore, in the
264 present study, the contents of two plant hormones were measured during grain filling.
265 Significant changes in the endogenous ABA and IAA contents in the grains were found
266 during grain filling (Figs. 5a and c). The ABA content increased during the early grain filling
267 stage until 12 DAF and then decreased until the end of grain filling. The endogenous ABA
268 content in the *qPE9-1* grains was always higher than that in *qpe9-1* (Fig. 5a). Very similar
269 patterns of dynamic changes in the IAA content were observed, with the *qPE9-1* grains
270 having higher IAA levels than *qpe9-1* during the grain-filling stages (Fig. 5c). The transcript
271 levels of rice *OsNCED5*, which encodes a key enzyme catalyzing ABA biosynthesis [9-cis-
272 epoxycarotenoid dioxygenase (*NCED*)], during grain filling were higher in *qPE9-1* than in
273 *qpe9-1* from 3 to 15 DAF (Fig. 5b). The change in the transcript level of one important IAA
274 biosynthesis gene (*OsTAR1*) was highly synchronized with changes in the grain IAA level
275 (Fig. 5d). These results suggest that ABA and IAA may be involved in grain-filling
276 regulation and that *qPE9-1* may exert its effects by changing the biosynthesis of these
277 hormones during grain filling.

278

279 ***qPE9-1* regulates starch biosynthesis in rice grains by ABA and IAA**

280 To verify the relationships between *qPE9-1* and the plant hormones ABA and IAA during the
281 grain-filling stages, exogenous ABA and IAA were applied to the developing grains during
282 the filling stage, and starch accumulation and gene expression were analyzed. As shown in
283 Fig. 6, all treatments had stimulatory effects on *OsSUS3*, *OsBEIIb* and *OsSSIIa* gene
284 expression and the activities of their encoded enzymes in the *qPE9-1* grains, but ABA
285 application had no effects on starch accumulation or gene expression in the *qpe9-1* grains
286 (i.e., ABA had either inhibitory or no effects on starch accumulation). Compared with *qpe9-1*

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287 grains, *qPE9-1* grains showed enhanced starch accumulation upon treatment with either
288 exogenous ABA or IAA, and this stimulatory effect of IAA was much more significant than
289 that of ABA (Fig. 6a). Application of IAA and ABA also stimulated *OsSUS3*, *OsBEIIb* and
290 *OsSSIIa* gene expression and the activities of their encoded enzymes in *qPE9-1* (Figs. 6b and
291 c). These results indicate that starch accumulation in *qPE9-1* grains is largely, if not
292 completely, dependent on high ABA and IAA levels.

293

294 Discussion

295 The rice *DEP1/qPE9-1* gene was formerly identified by two independent research groups as a
296 gene that controls panicle erectness, grain number per panicle and, consequently, grain yield
297 (Huang *et al*., 2009; Zhou *et al*., 2009). Sequential alignment at the amino acid level showed
298 that *qPE9-1* was a homolog of *AGG3*, which is a γ subunit of the *Arabidopsis* heterotrimeric
299 G protein, although its similarity and degree of homology were not as high. AGG3 is a
300 recently discovered novel protein that is almost twice as large as a typical G_γ protein. This
301 protein exhibits a high degree of similarity with canonical G_γ proteins at the N-terminus,
302 whereas the C-terminus is plant-specific and contains an extremely high number of cysteine
303 residues (Chakravorty *et al*., 2011; Botella, 2012). In plants, the G_γ and G_β subunits are
304 tightly bound physically and exert their effects as a dimer in both the active and inactive
305 states of the heterotrimeric G protein. Recent studies showed that based on the interaction
306 between DEP1/qPE9-1 and RGB1 (G_β subunit of rice), DEP1/qPE9-1 is a G_γ protein in rice
307 (Botella, 2012; Sun *et al*., 2014).

308 The functions and molecular mechanisms of DEP1/qPE9-1 as a new G_γ subunit in rice are
309 largely unknown. Because *DEP1/qPE9-1* has been cloned and identified as a gene controlling
310 the panicle size and rice shape (Huang *et al*., 2009; Zhou *et al*., 2009), we can reasonably

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311 assume that *DEP1/qPE9-1* may play important roles in regulating grain development and the
312 grain-filling process. Rice grain formation and grain filling are rather complicated processes
313 that involve approximately 21,000 genes, including 269 genes that are closely related to
314 various physiological and biochemical pathways (Zhu *et al.*, 2003). Proteomic research
315 indicates that 123 proteins and 43 phosphoproteins are involved in regulation of the grain-
316 filling process (Zhang *et al.*, 2014). Furthermore, the grain-filling process is also regulated by
317 plant hormones (e.g., ABA, IAA, cytokinin and ethylene), which fluctuate considerably
318 during the grain-filling period (Yang *et al.*, 2001; 2003; 2006; Tang *et al.*, 2009). Although
319 the carbohydrate interconversion biochemical pathway has clearly been elucidated during
320 grain filling, the biochemical and molecular mechanisms controlling rice grain size and
321 weight are largely unknown.

322 Overexpression of *Arabidopsis* atypical G_γ (*AGG3*) promotes seed and organ growth by
323 increasing cell proliferation, and *AGG3*-mutated lines have a small seed size (Chakravorty *et*
324 *al.*, 2011; Li *et al.*, 2012; Roy Choudhury *et al.*, 2013). Similar results have been reported for
325 rice plants. *GRAIN SIZE 3* (*GS3*) and *DENSE AND ERECT PANICLE1* (*DEP1*)/*PANICLE*
326 *ERECTNESS* (*qPE9-1*) are involved in controlling either the grain size or panicle size in rice
327 (Fan *et al.*, 2006; Huang *et al.*, 2009; Zhou *et al.*, 2009). However, in contrast to *Arabidopsis*,
328 *GS3* negatively regulates the grain size, whereas *DEP1/qPE9-1* positively regulates both the
329 grain and panicle size (Fan *et al.*, 2006; Huang *et al.*, 2009; Zhou *et al.*, 2009). Why this
330 considerable difference exists between these plant species remains unclear and should be
331 studied and elucidated in the future.

332 A comparison of the panicle morphology, grain size and final grain weight clearly
333 indicated that *qpe9-1* had a smaller size and lower grain weight than *qPE9-1*, implying that
334 *qPE9-1* positively regulated the grain size and final grain weight (Fig. 1). Our previous
335 research and that conducted by other groups revealed that the grain size and final grain

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336 weight were largely determined by two factors/processes: the number and size of endosperm
337 cells (i.e., the sink size) and the grain-filling process, including the grain-filling rate and
338 duration (i.e., the sink activity; Liang *et al.*, 2001). The smaller grain size and lower final
339 grain weight of *qpe9-1* are a result of fewer endosperm cells and lower starch accumulation
340 after flowering, the latter is partly due to reducing the duration of the grain-filling process
341 (Figs. 2 and 3). However, the mechanisms underlying how *qPE9-1* controls endosperm cell
342 proliferation and starch accumulation are unknown.

343 More than ten proteins/enzymes are directly involved in the biochemical pathways of
344 carbohydrate interconversion and starch biosynthesis during rice grain filling (Tetlow *et al.*,
345 2004; Ohdan *et al.*, 2005; Zhu *et al.*, 2011). What is the underlying cause of lower starch
346 accumulation in *qpe9-1* aside from the lower numbers of endosperm cells? In other words, do
347 significant differences exist in the expression of key protein/enzyme-encoding genes or the
348 activity of these key enzymes between the two genotypes? To answer these questions, we
349 compared differences in the expression of genes encoding several key enzymes that catalyzed
350 carbohydrate interconversion and starch biosynthesis between the two genotypes used in this
351 experiment. Our results clearly indicate that the lower expression levels of several key genes
352 that encode enzymes catalyzing starch biosynthesis and the lower activities of these enzymes
353 in the *qpe9-1* grains during the late grain filling stage are the most important factors resulting
354 in the lower grain weight (Fig. 4).

355 The grain-filling process is a highly regulated process that involves both genetic and
356 environmental factors. Plant hormones play important roles in grain growth and development
357 (Tang *et al.*, 2009; Zhu *et al.*, 2011; Zhang *et al.*, 2016). However, little is known about the
358 detailed mechanisms by which plant hormones regulate the grain-filling process. Yang *et al*.
359 (2001) suggested that an altered hormonal balance, especially a decrease in GA and an
360 increase in ABA, enhanced the remobilization of prestored carbon to the grains and

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361 accelerated the grain-filling rate. In addition, IAA treatment increased spikelet growth and
362 development in distal branches (Patel & Mohapatra, 1992). Recently, studies in peas
363 provided direct evidence that auxin was required for a normal seed size and starch
364 accumulation. The mutant of *TAR2*, which is an IAA biosynthesis gene, induces the
365 formation of small seeds with reduced starch content and a wrinkled phenotype at the dry
366 stage. Application of the synthetic auxin 2,4-D partially reversed the wrinkled phenotype but
367 did not restore the starch content of the mutant seeds to that of the WT (McAdam et al.,
368 2017). Our results showed considerable differences in the dynamics of the endogenous
369 hormone levels in the grains during grain filling, and the endogenous ABA and IAA levels
370 were positively related to grain filling during the entire grain-filling stage (Fig. 6). The levels
371 of these endogenous plant hormones were significantly lower in the *qpe9-1* than in the *qPE9-*
372 *1* grains during the grain-filling stages (Figs. 5a and c). Based on the effects of exogenous
373 ABA and IAA on starch accumulation and the expression of genes that encode several key
374 enzymes catalyzing starch biosynthesis, we conclude that the positive control of *qPE9-1* on
375 the grain-filling process occurs largely through the biosynthesis of endogenous plant
376 hormones.

377 Rice grain filling is a very complicated process that involves photoassimilate (mainly
378 sucrose) translocation from photosynthetic sources (i.e., leaves and leaf sheaths), sucrose
379 degradation, transmembrane transport and starch synthesis in the grains (i.e., the sink, Liang
380 *et al.*, 2001; Lü *et al.*, 2008; Tang *et al.*, 2009). Approximately twenty enzymes/proteins have
381 been reported to be involved in these biochemical processes (Tetlow *et al.*, 2004; Ohdan *et*
382 *al.*, 2005; Zhu *et al.*, 2003). However, the mechanism of starch biosynthesis regulation in
383 grains is not well understood. This research showed that the novel G protein γ subunit *qPE9-*
384 *1* increased the numbers of endosperm cells and positively regulated starch biosynthesis,
385 which enhanced the grain size and weight. *qPE9-1* also enhanced the accumulation of ABA

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386 and IAA. In turn, these hormones regulate the expression of genes encoding several key
387 enzymes that catalyze starch biosynthesis during the late grain filling stage and consequently
388 affect the final grain weight.

389

390 Supplementary Data

391 **Figure S1.** The evaluation of antibody valence. **a** Analysis of the RGB1 protein using a
392 RGB1 antibody; **b** Analysis of a peptide fragment of the DEP1/qPE9-1 protein using a
393 DEP1/qPE9-1 antibody; **c** Detection of the His-tagged DEP1/qPE9-1 protein using both
394 DEP1/qPE9-1 and His tag antibodies.

395

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399

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484

485 ## Figure Legends

486 **Figure 1. Grain performance of *qpe9-1* and *qPE9-1*. a** Grain length; **b** Grain width; **c**
487 Brown rice during grain development; **d** qPE9-1 protein expression in grains at 5 DAF was
488 analyzed by incubating isolated proteins with polyclonal antibodies against qPE9-1 or RGB1
489 (as a loading control); **e** *qPE9-1* gene expression was monitored in grains at 5 DAF using
490 qRT-PCR (n=3); **f** Comparisons between *qpe9-1* and *qPE9-1* with respect to the grain length,
491 width and thickness; **g** The 1000-grain weight (n=3). DAF, days after flowering. Data are
492 presented as the mean ± SD. "*" represents a significant difference at P <0.05.

493

494 **Figure 2. Histological analyses of endosperms at maturity and changes in endosperm**
495 **size after fertilization in *qpe9-1* and *qPE9-1*. a** Cross-sections of the endosperm between
496 the dorsal and central points showing the cell sizes and numbers. Scale bars, 100 µm; **b**
497 Comparison of cell numbers in the endosperm cross-sections (n=5 endosperms from 5
498 panicles); **c** Changes in the numbers of endosperm cells during grain filling (n=10
499 endosperms from 5 panicles); **d** The long and short axes of endosperm cells during grain
500 development. Data are presented as the mean ± SD. "*" represents a significant difference at
501 $P < 0.05$.

502

503 **Figure 3. Starch accumulation during grain filling. a** Starch accumulation of grains during
504 the grain-filling stages (n=5); **b** The rate of starch accumulation during grain filling; **c** The

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505 total starch content in the flour (n=5). Data are presented as the mean ± SD. "*" represents a

506 significant difference at $P < 0.05$.

507

508 **Figure 4. Expression of several starch biosynthesis genes and changes in the activities of**

509 **these enzymes during grain filling. a** *OsSUS3*, *OsSSIIa* and *OsBEIIb* expression levels

510 during grain filling (n=3); **b** Changes in the SUS, SS and BE activities during grain filling

511 (n=3), 1 U= 1 μg/min/mg protein. Data are presented as the mean ± SD. "*" represents a

512 significant difference at $P < 0.05$.

513

514 **Figure 5. Changes in the ABA and IAA contents and the expression of genes encoding**

515 **key enzymes catalyzing their biosynthesis during grain filling. a** Changes in the ABA

516 level in rice endosperm cells during seed development (n=3); **b** Changes in the expression

517 profile of the ABA synthesis gene *OsNCED5* in rice endosperm cells during seed

518 development (n=3); **c** Changes in the IAA levels in rice endosperm cells during seed

519 development (n=3); **d** Changes in the expression profile of the IAA synthesis gene *OsTAR1*

520 in rice endosperm cells during seed development (n=3). Data are presented as the mean ± SD.

521 "*" represents a significant difference at $P < 0.05$.

522

523

524 **Figure 6. Effects of exogenous ABA and IAA applications on starch biosynthesis. a**

525 Effects of exogenous ABA and IAA on starch accumulation (n=5); **b** Effects of exogenous

526 ABA and IAA on the SUS, SS and BE activities at 21 DAF (n=3), 1 U= 1 μg/min/mg protein;

527 **c** Effects of exogenous ABA and IAA on *OsSUS3*, *OsSSIIa* and *OsBEIIb* expression at 21

528 DAF (n=3). Data are presented as the mean ± SD. "*" represents a significant difference at P

529 < 0.05.

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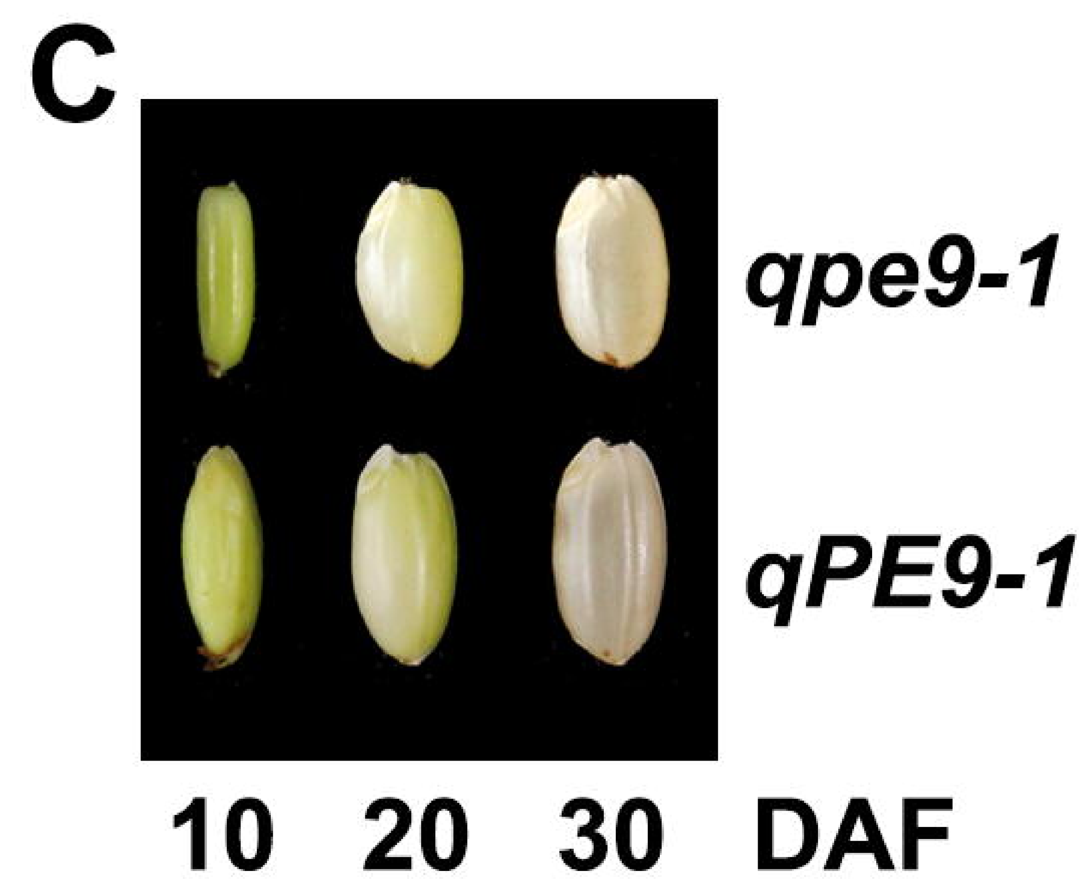

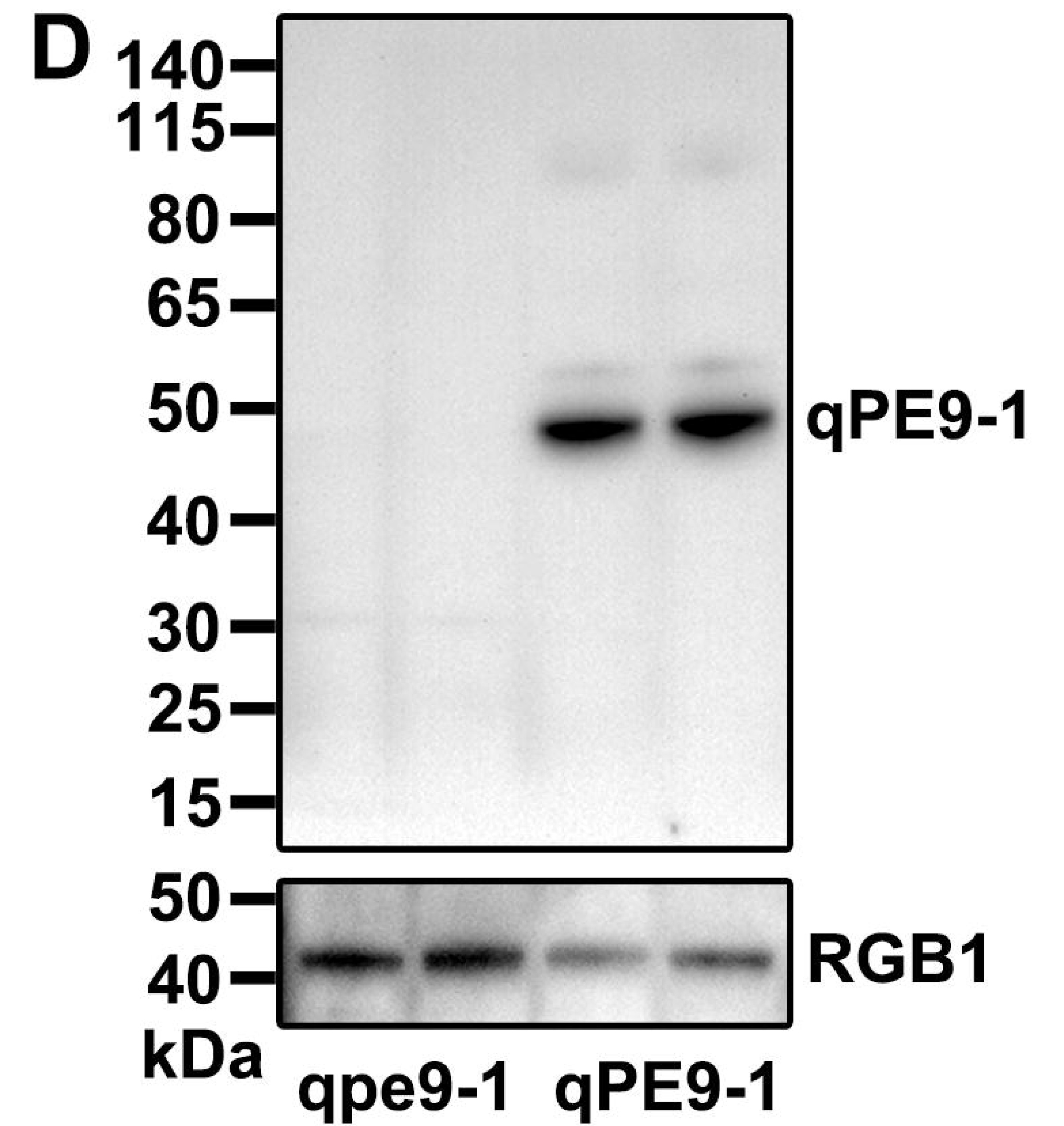

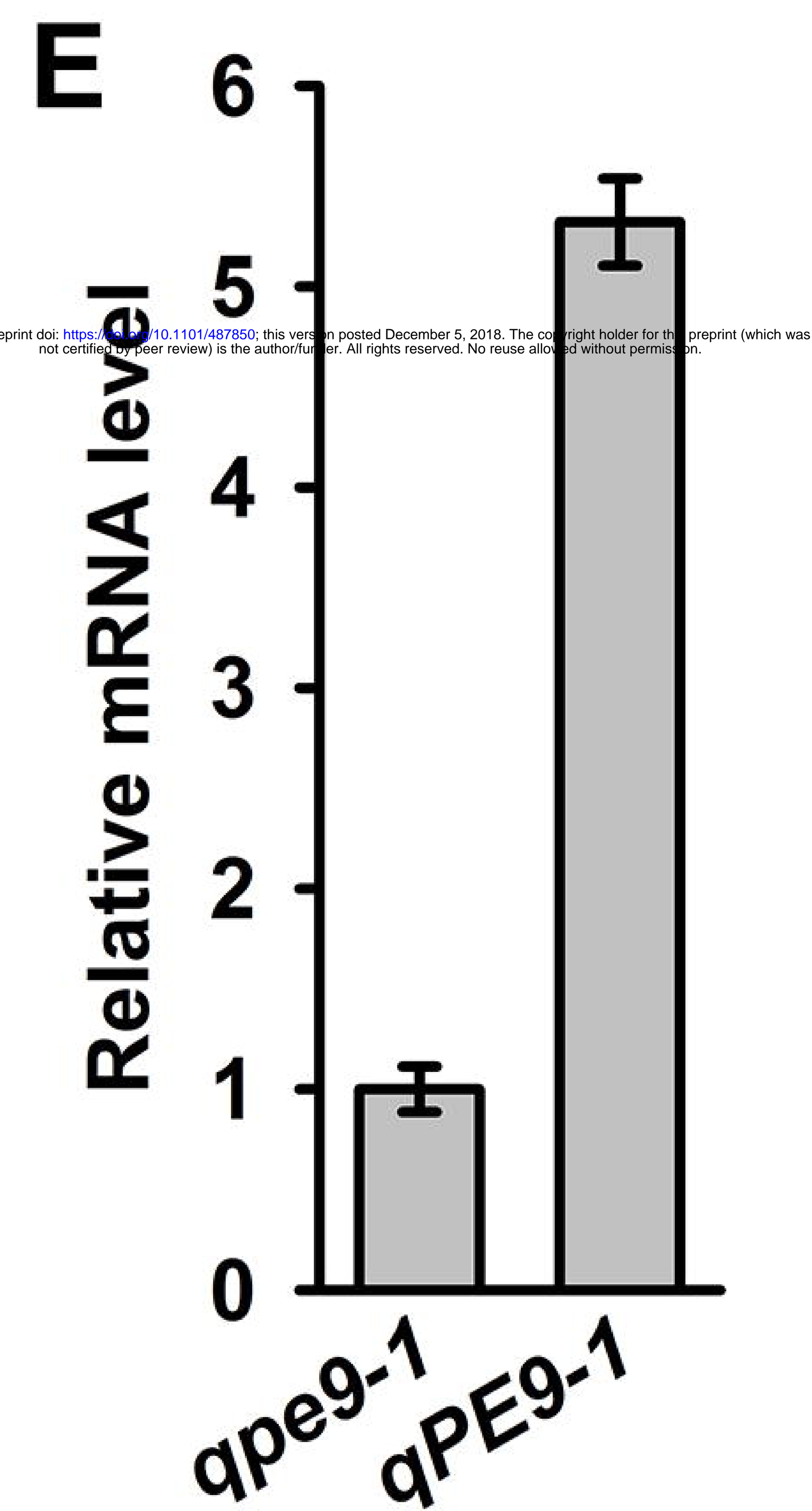

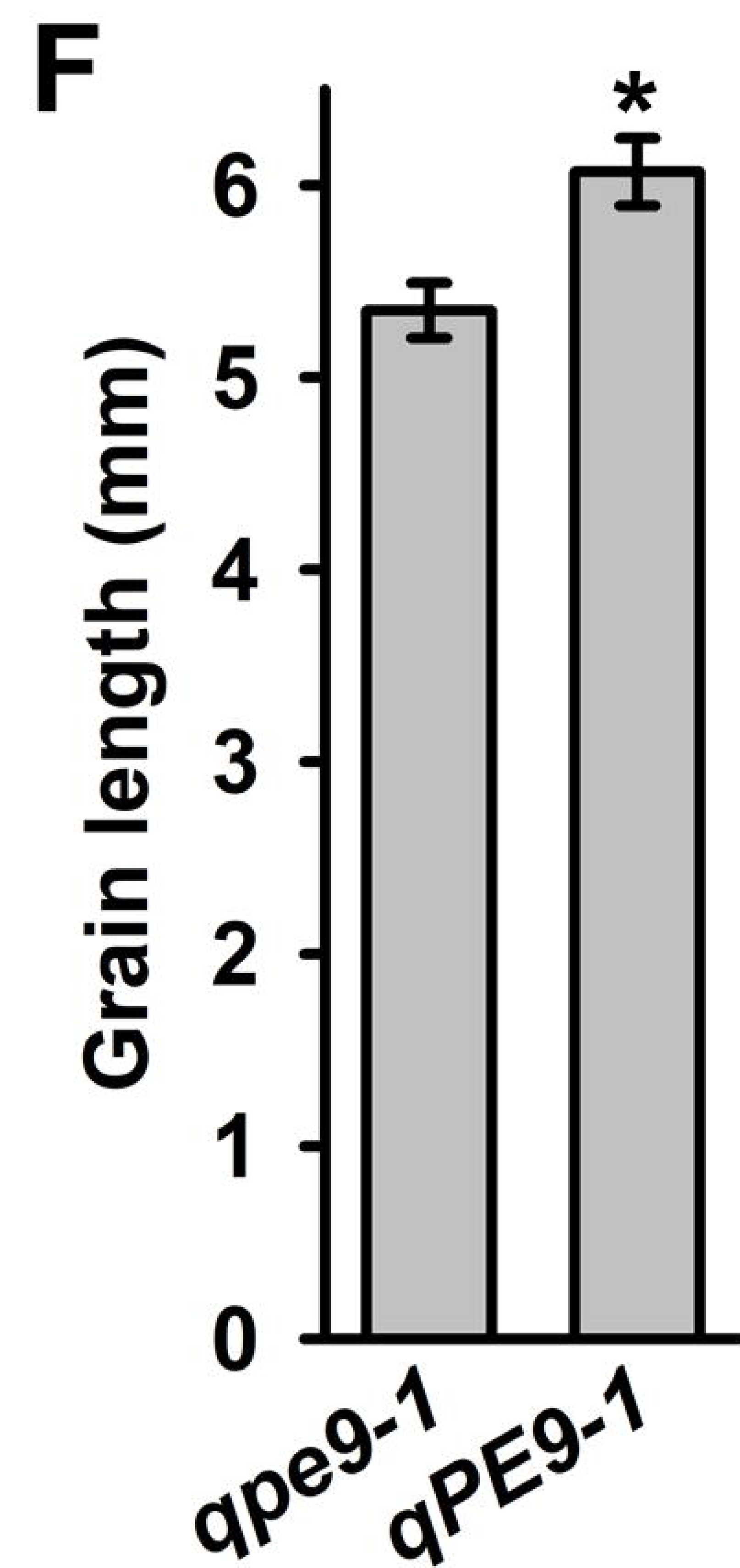

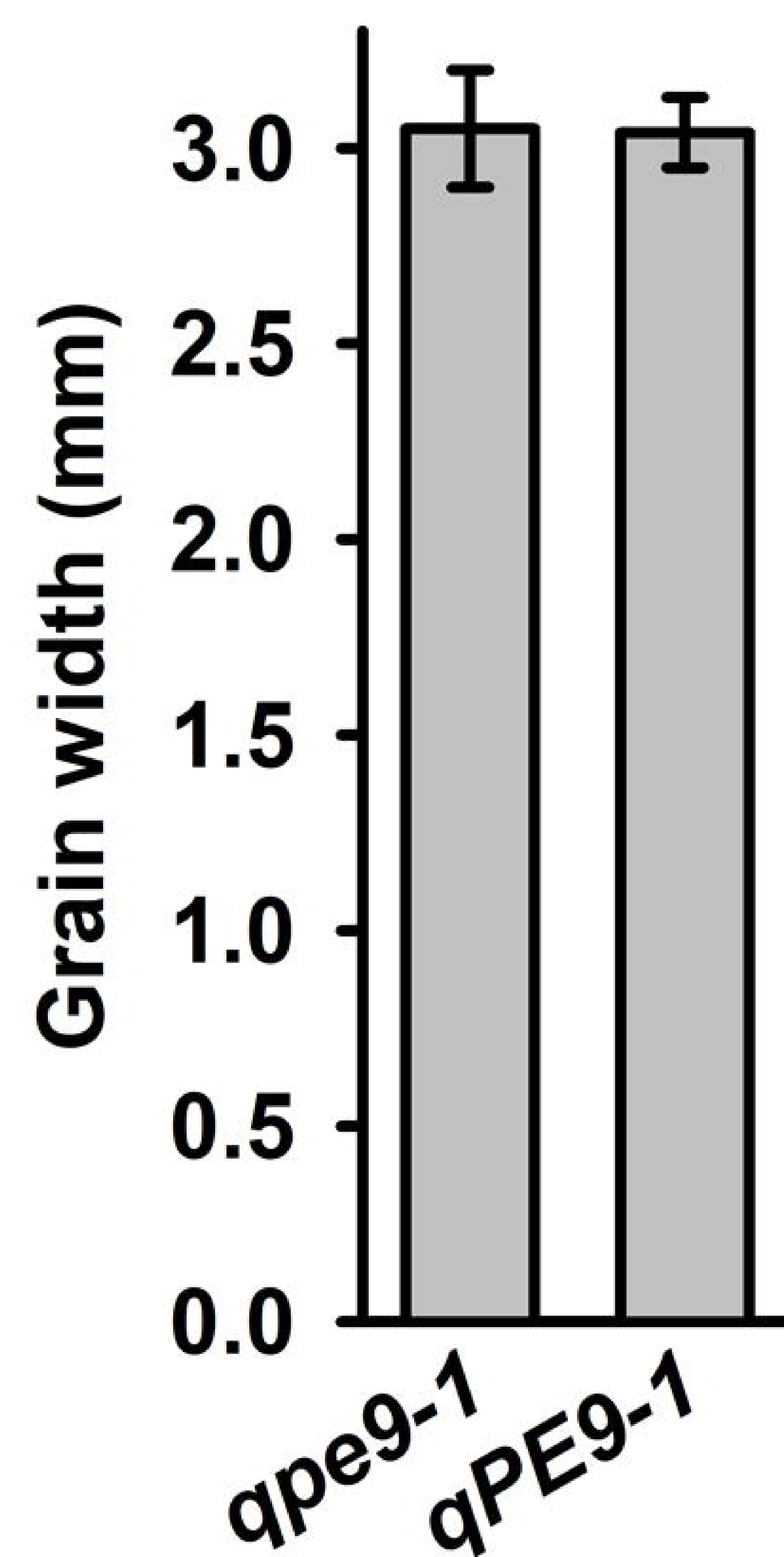

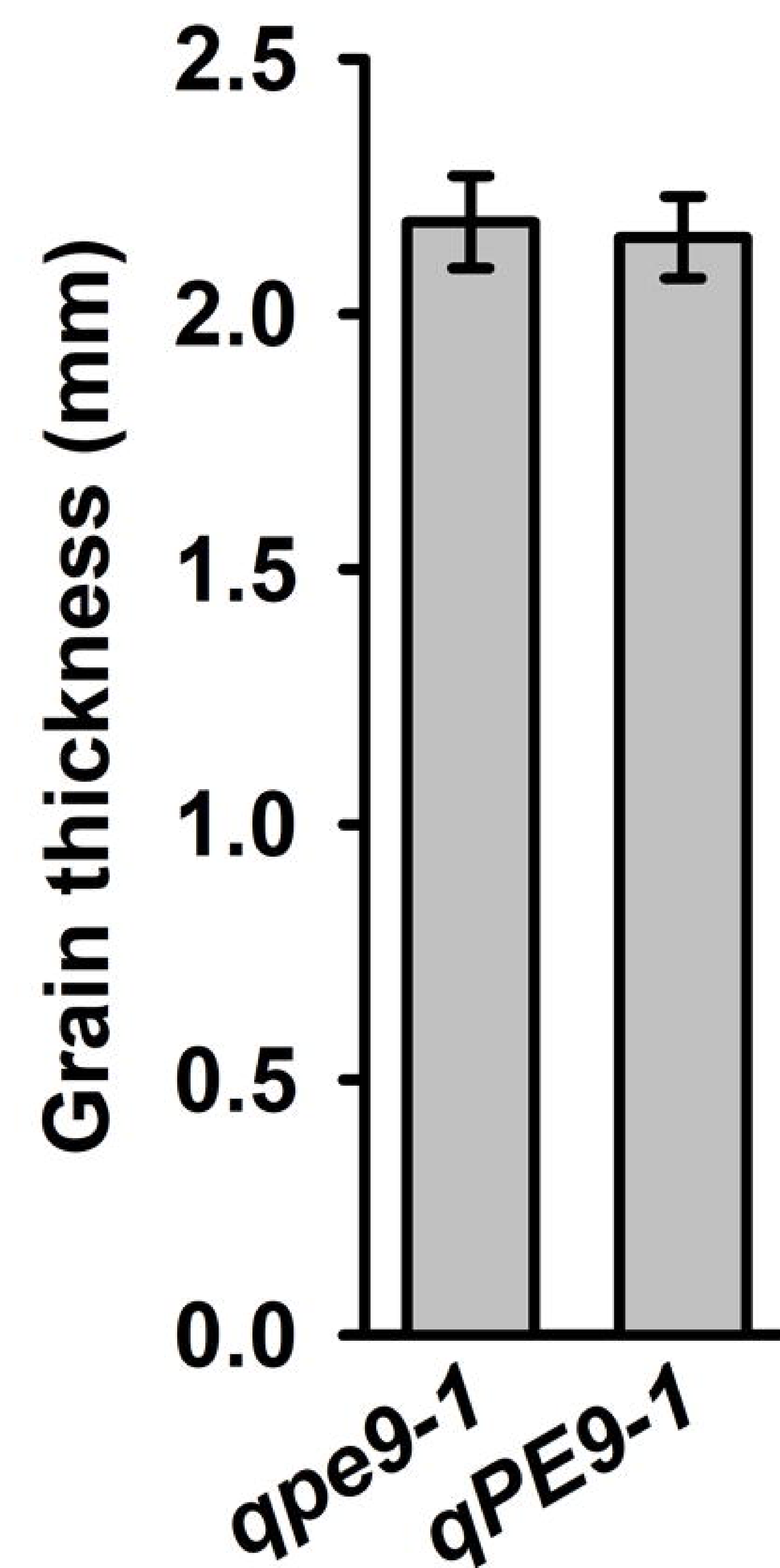

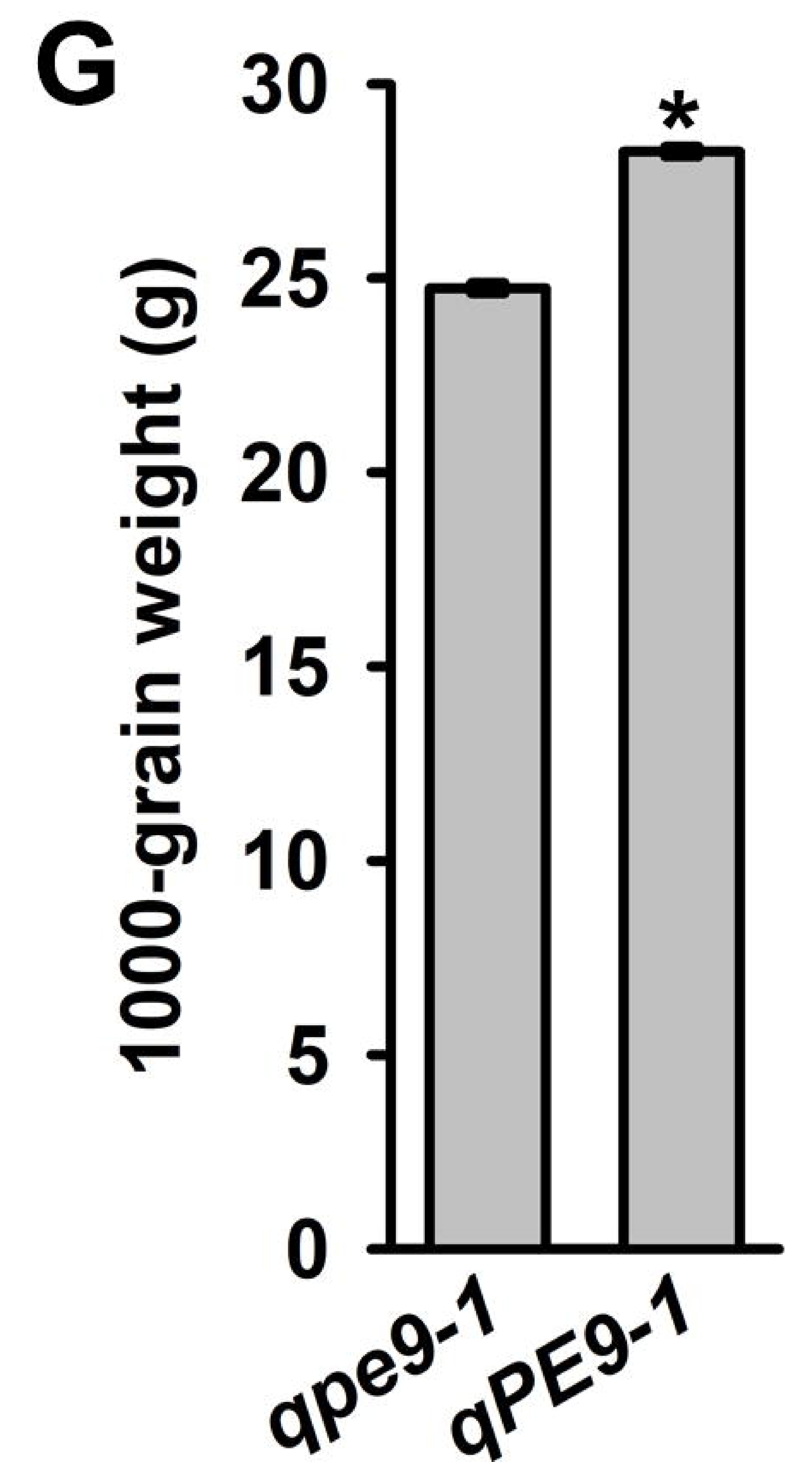

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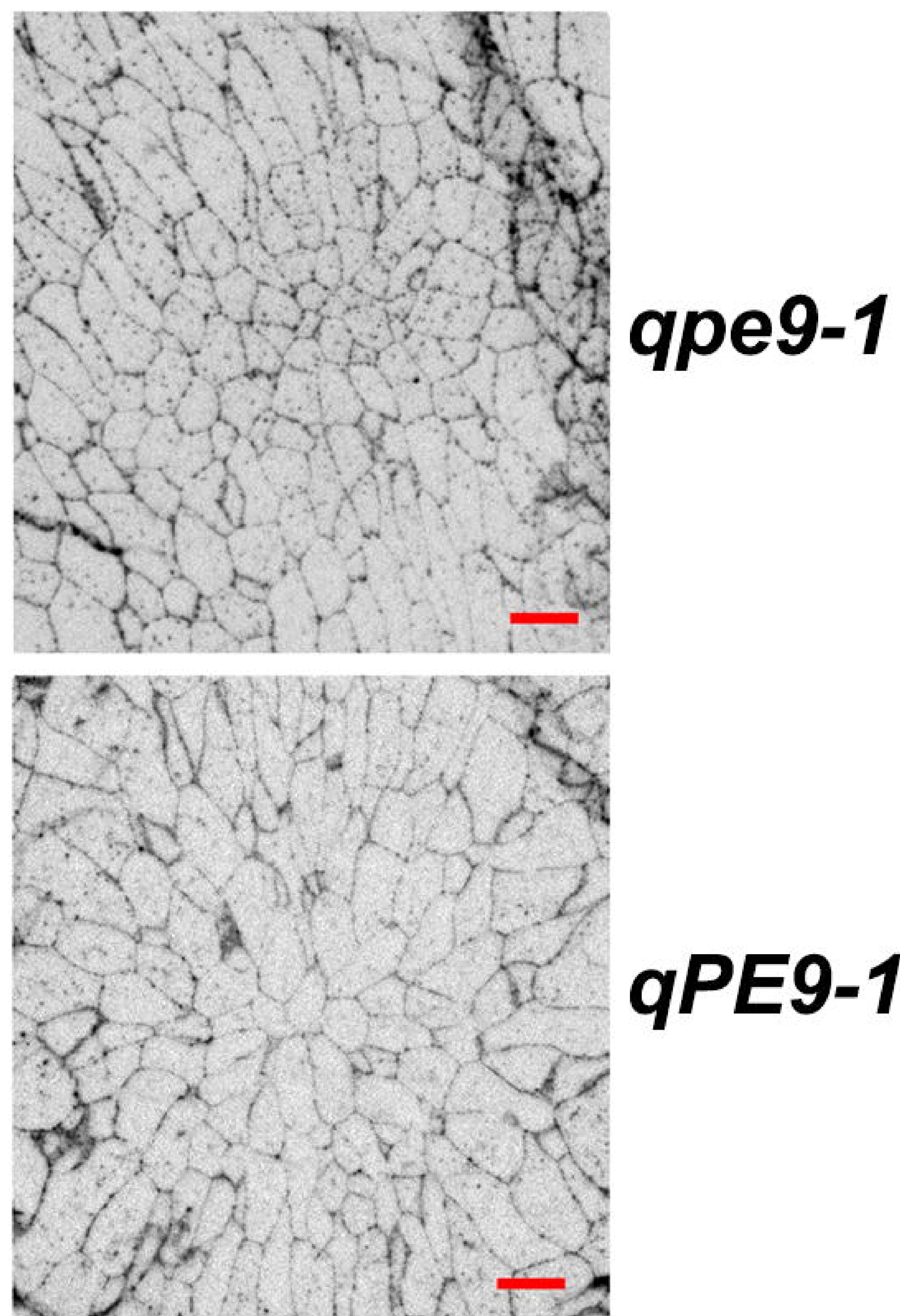

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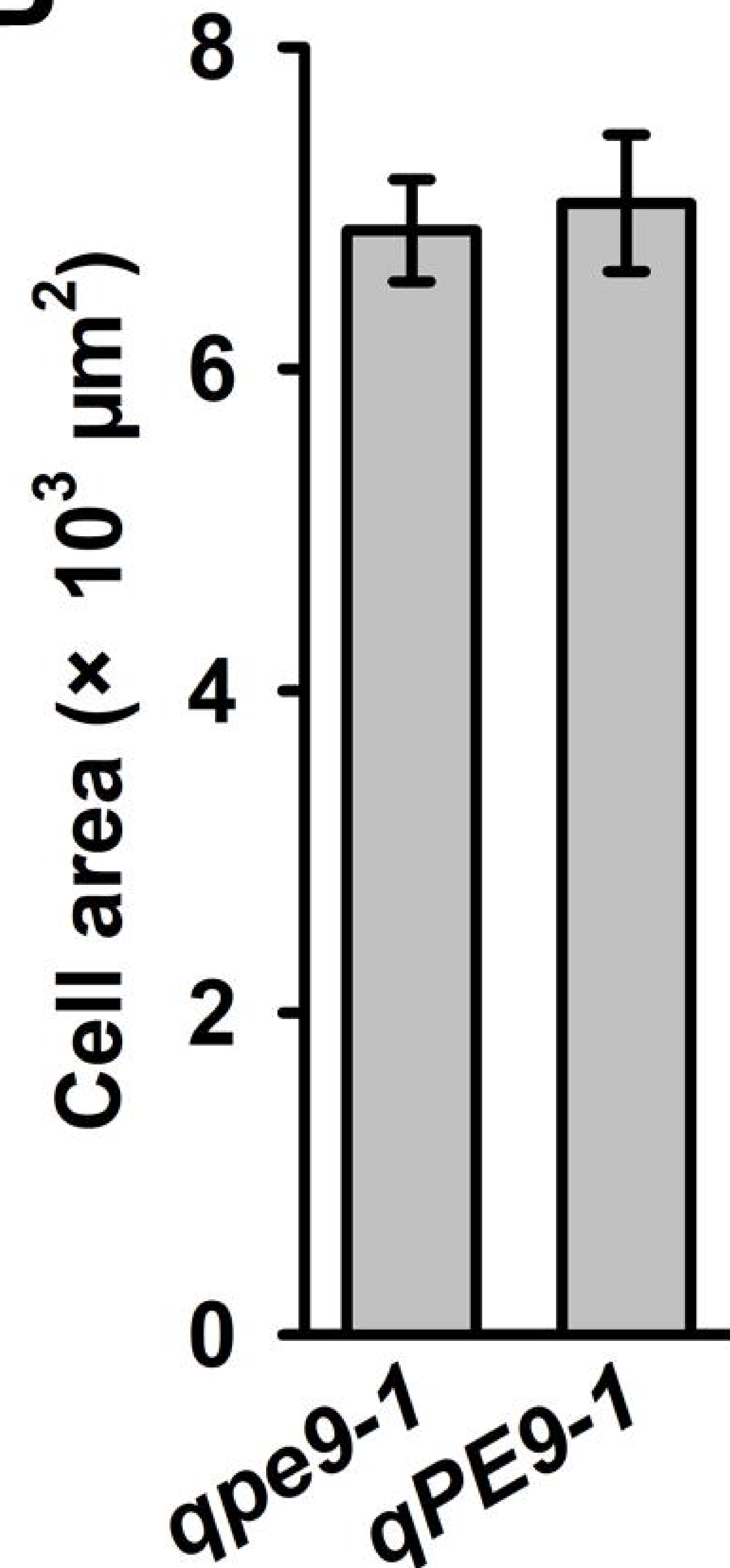

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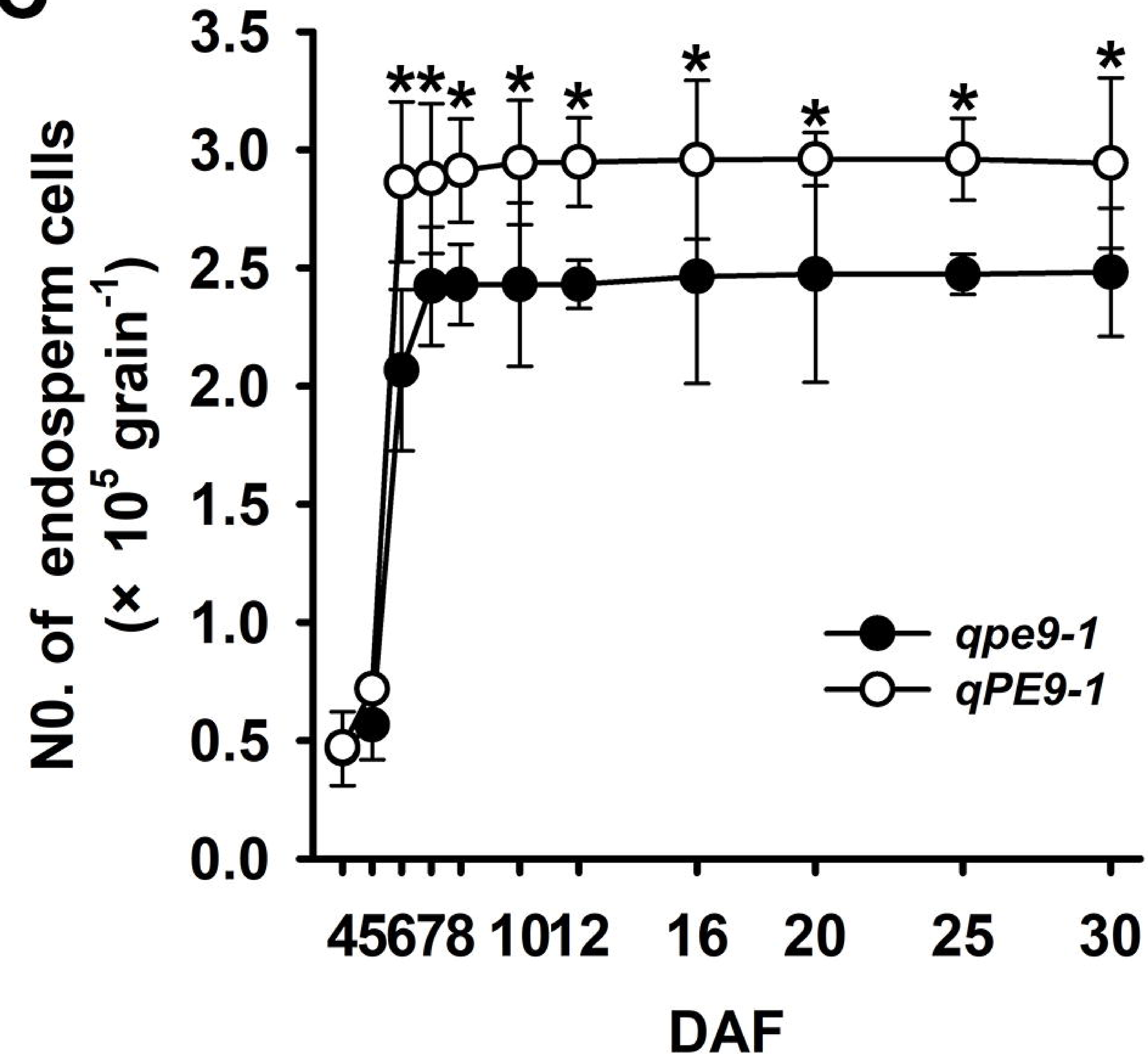

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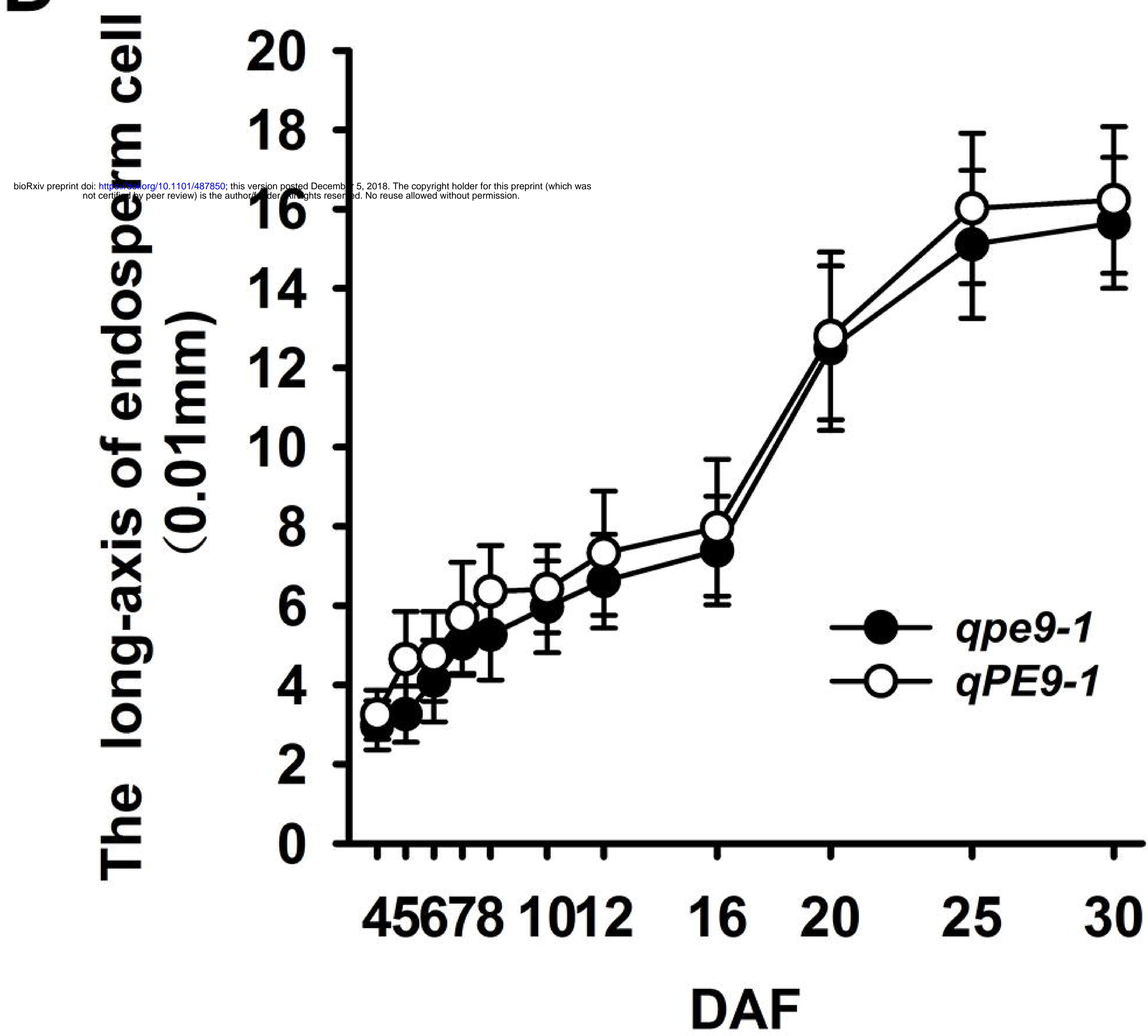

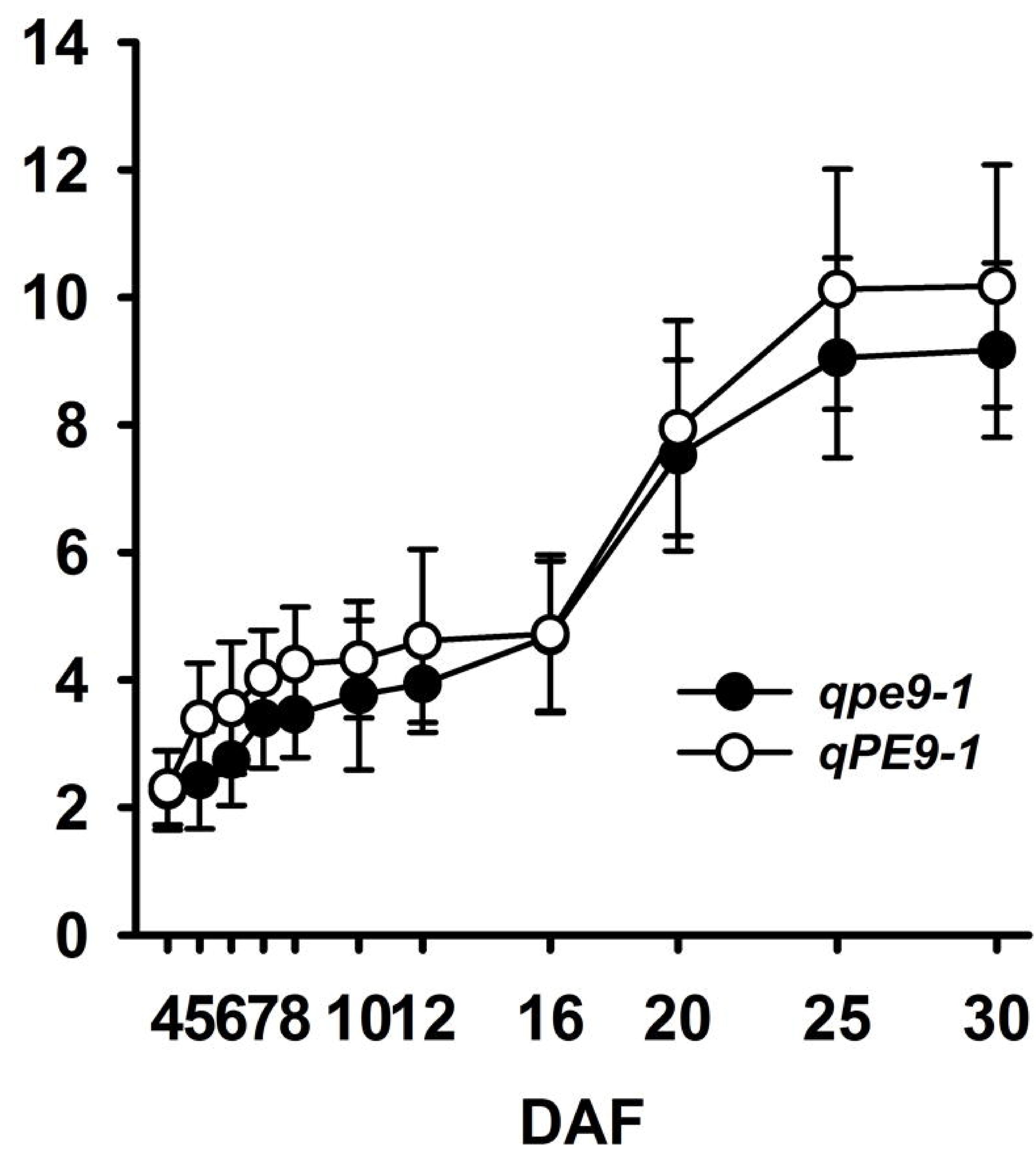

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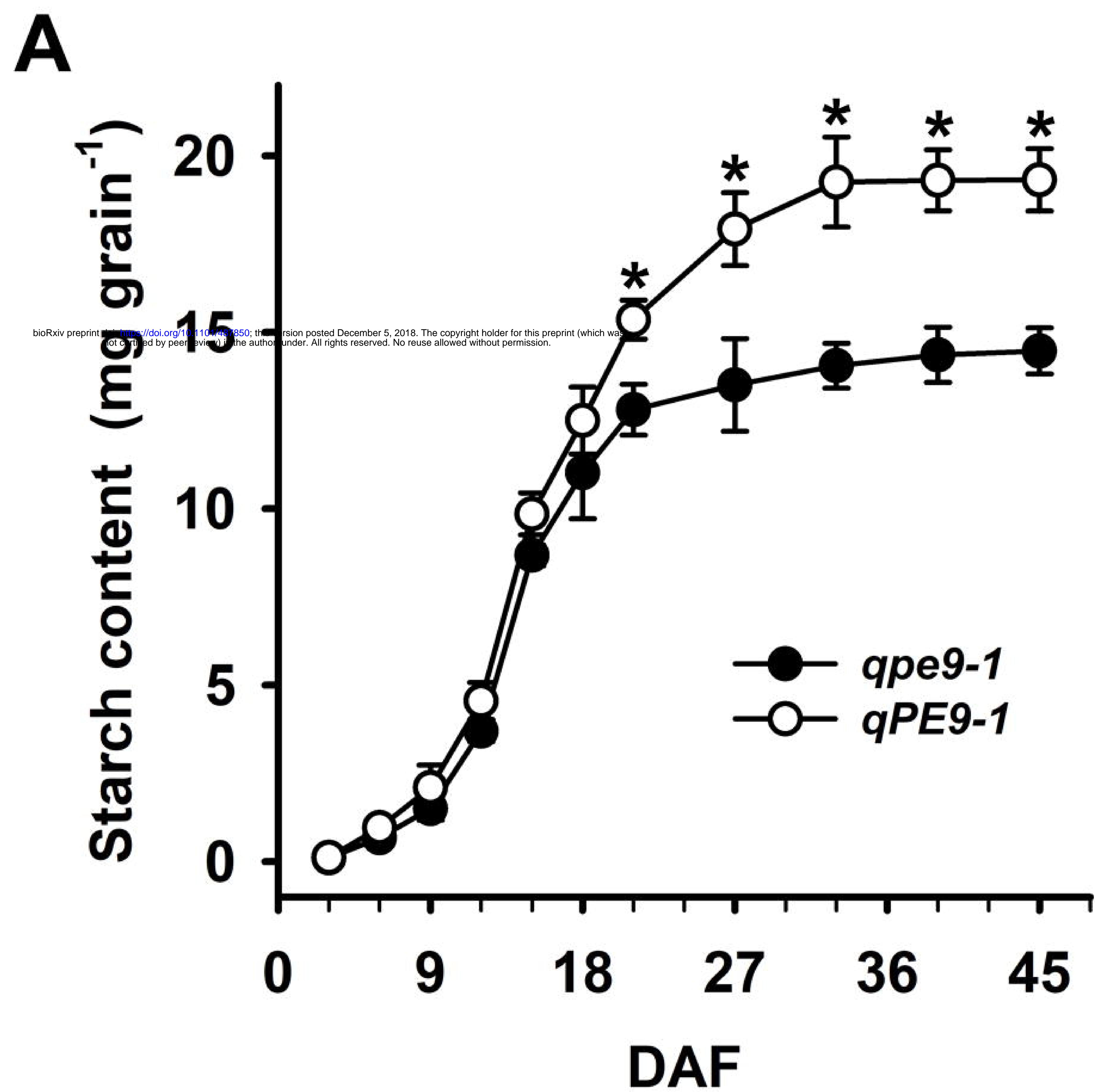

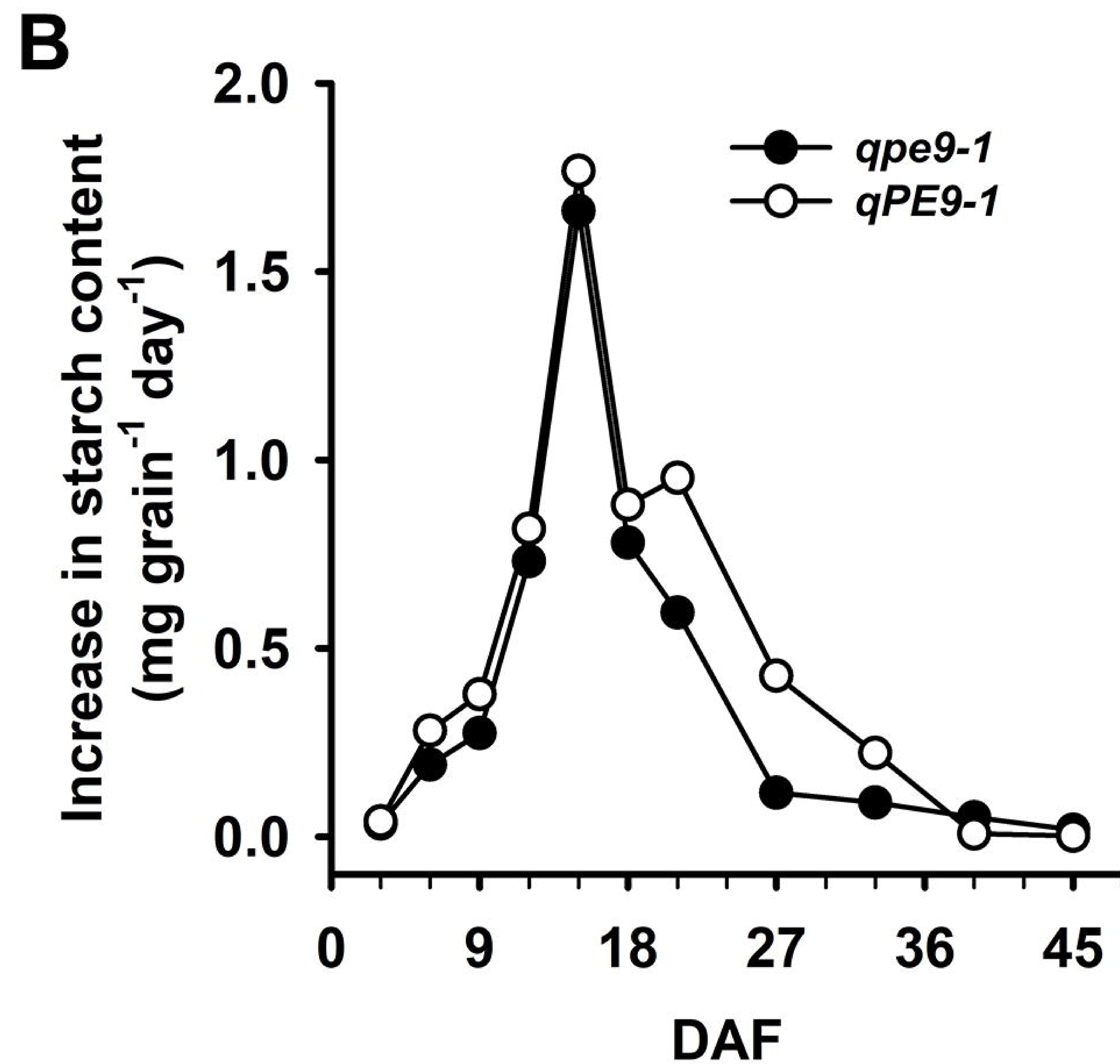

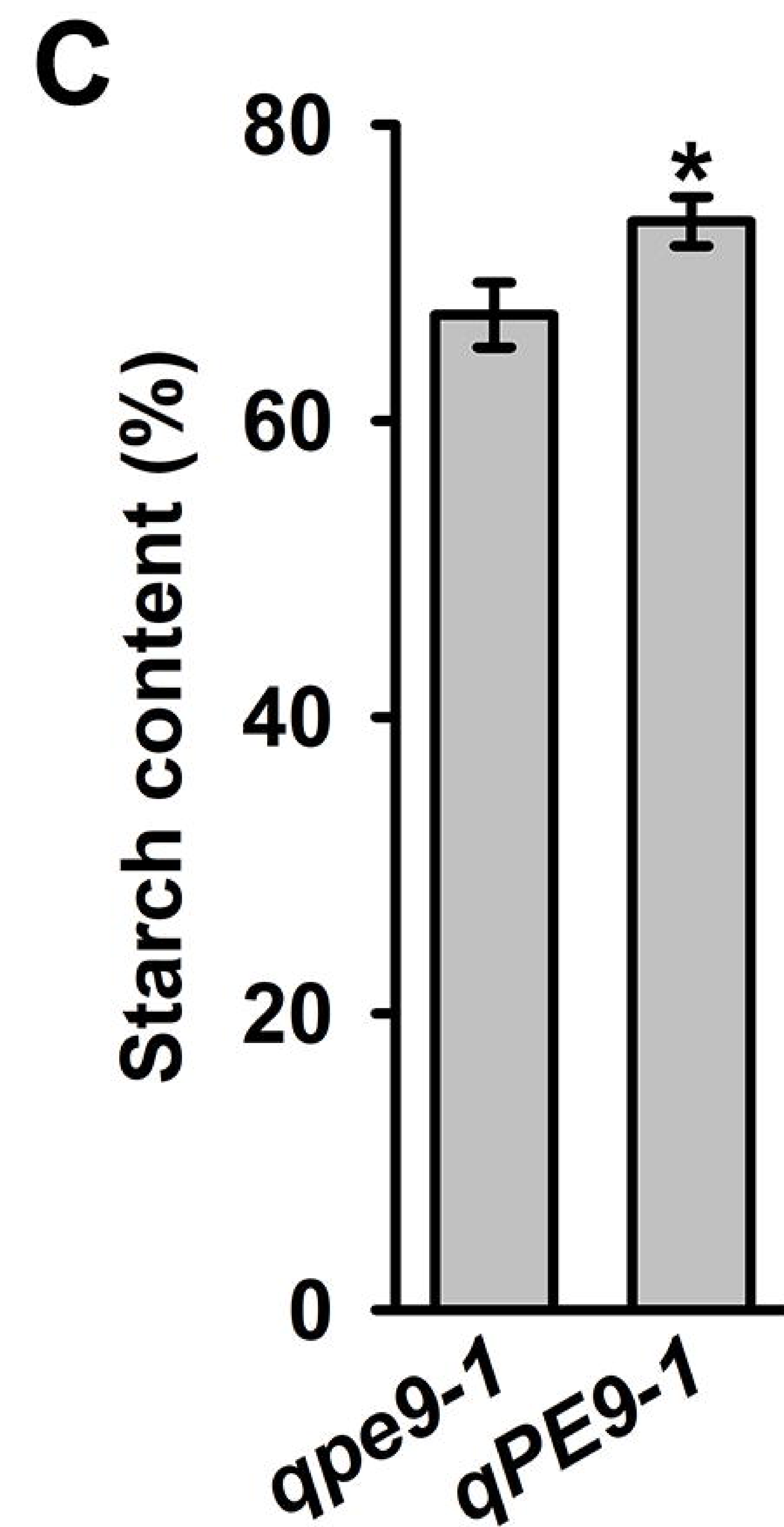

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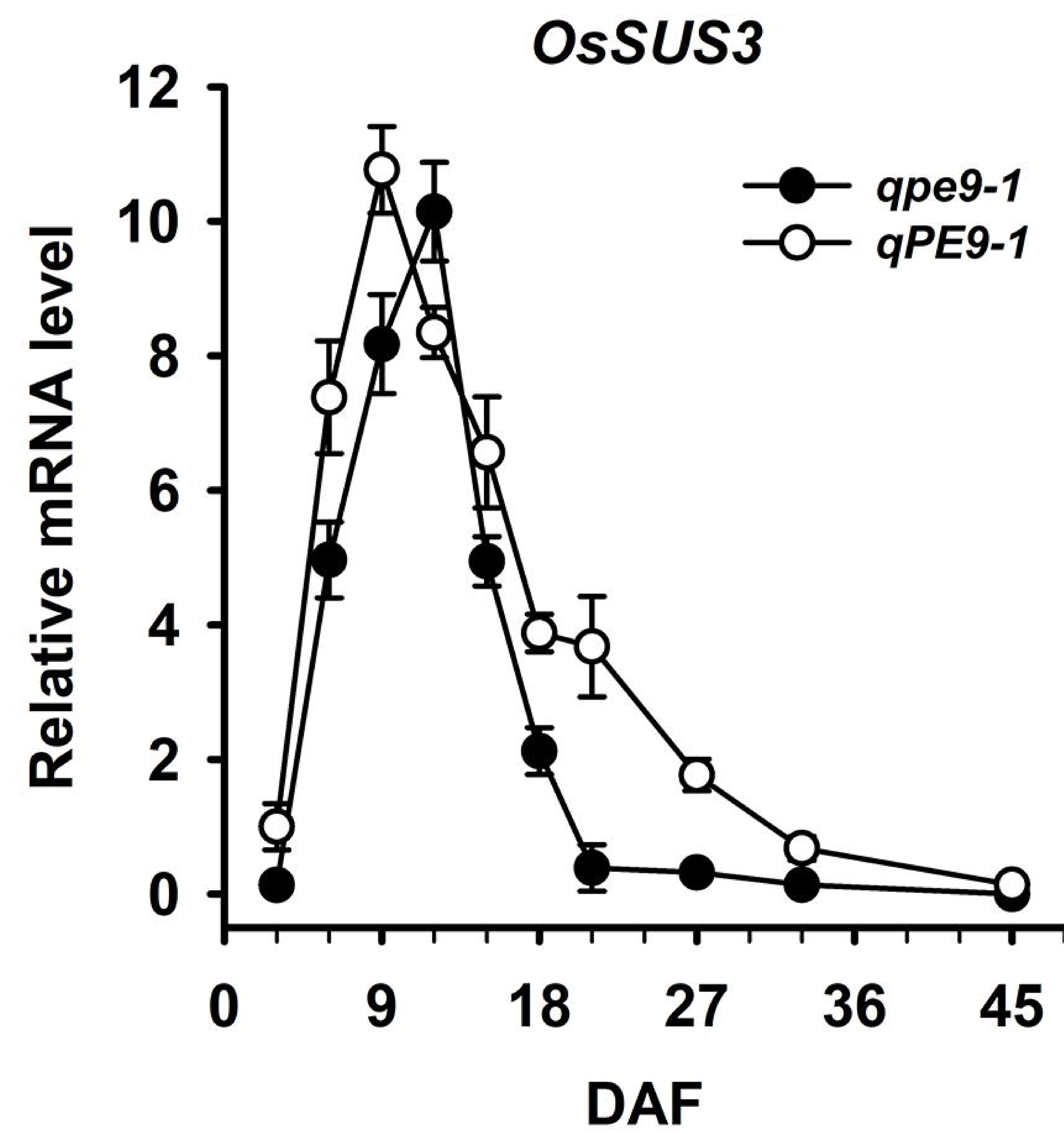

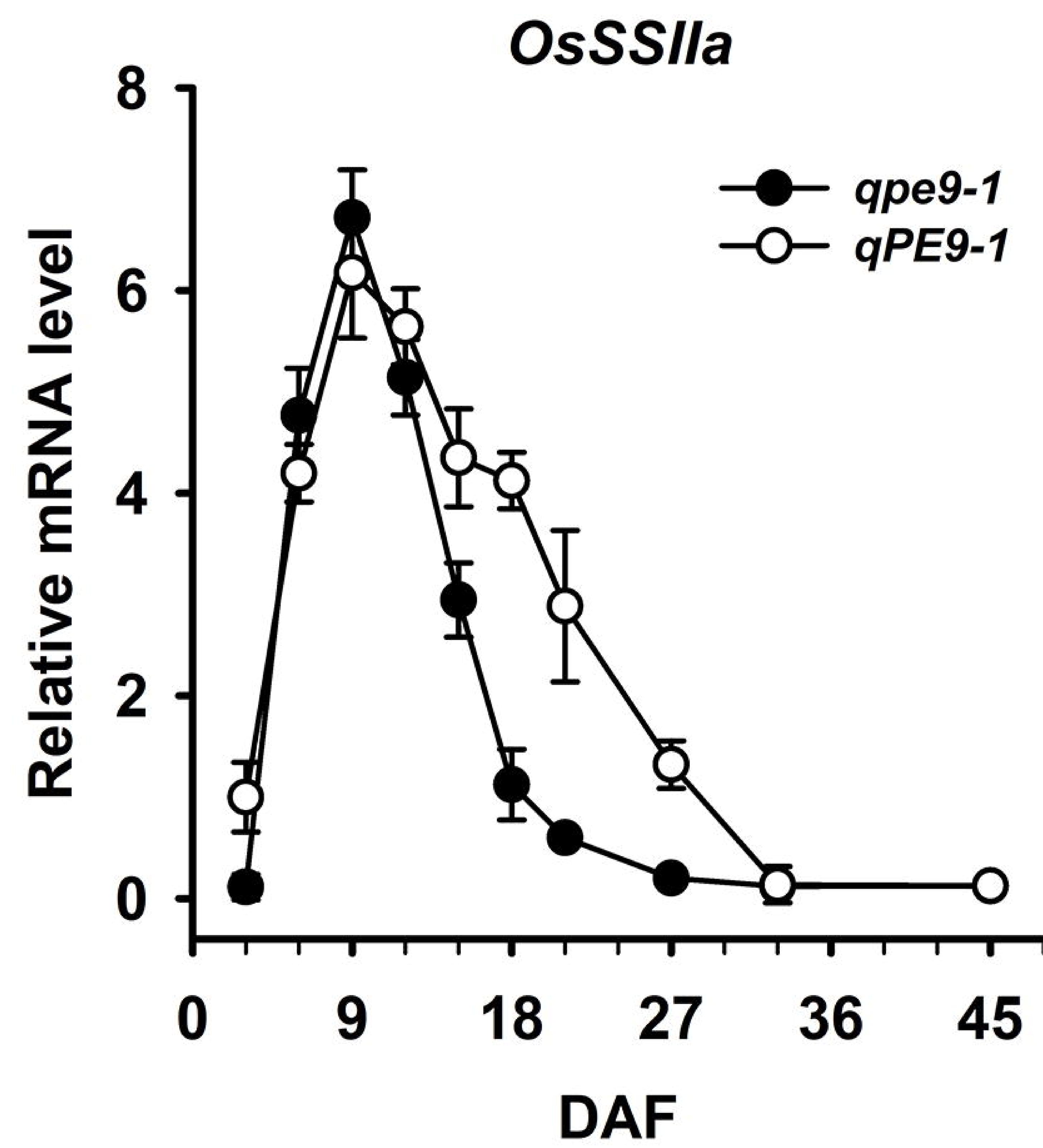

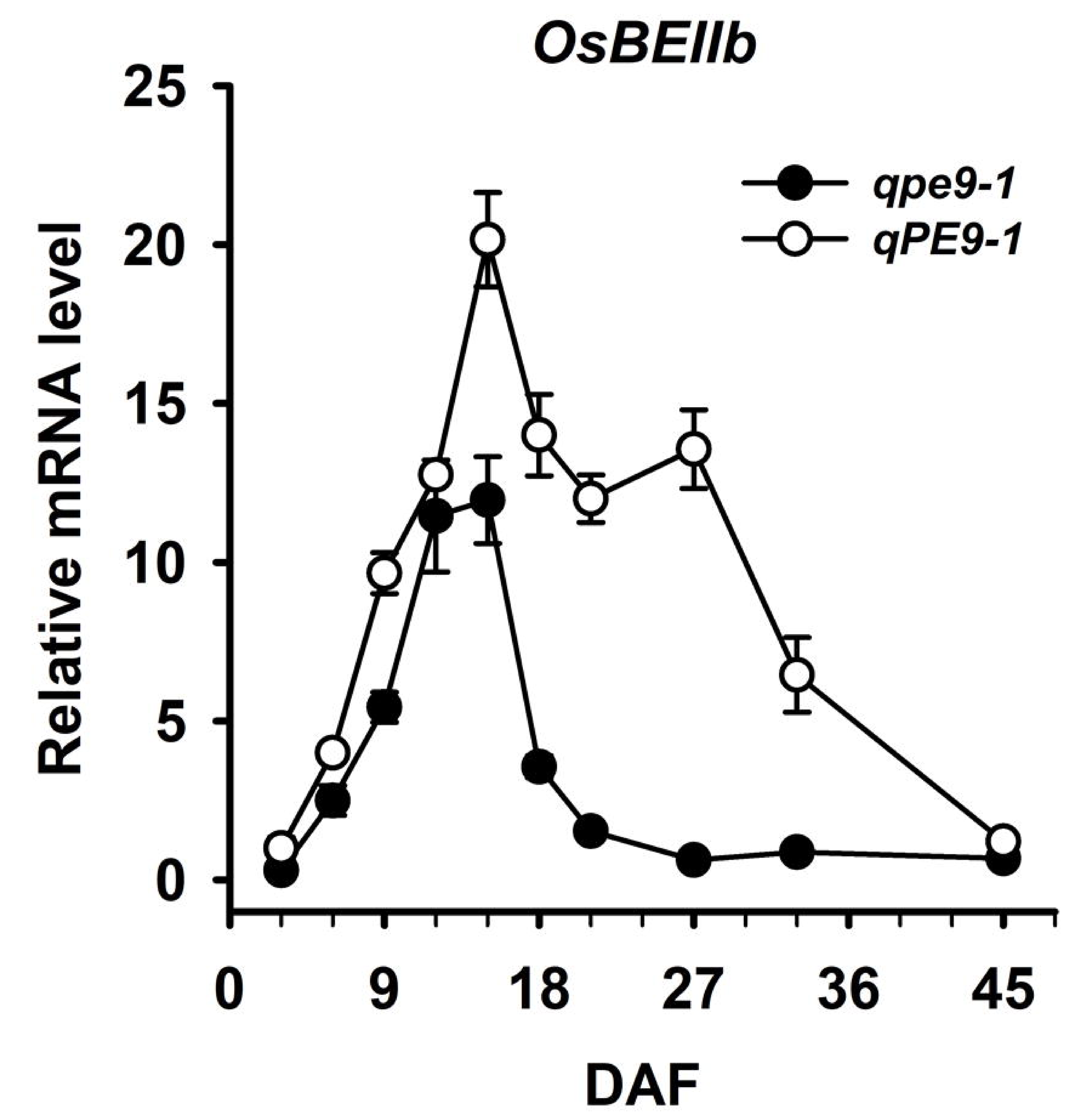

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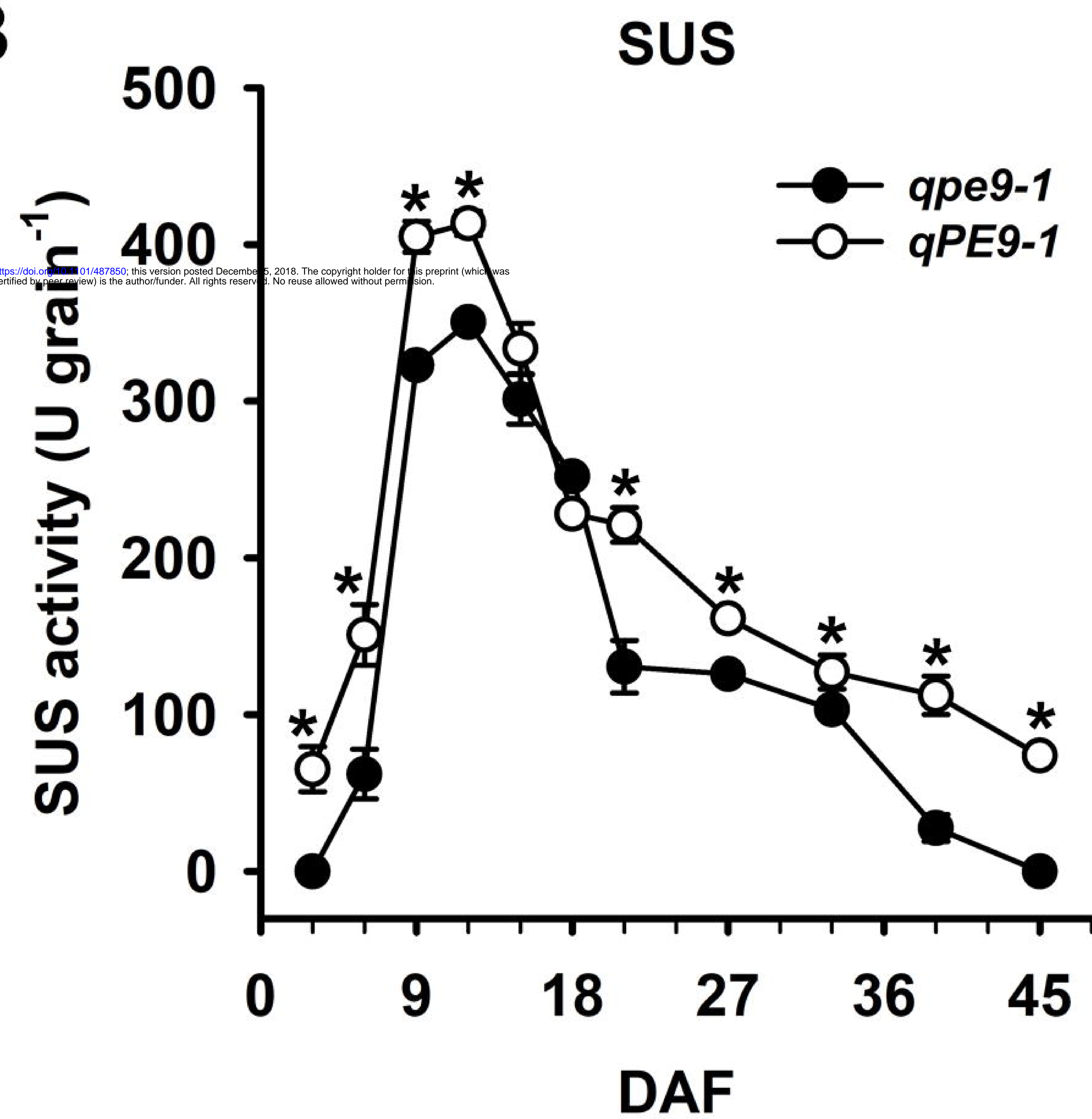

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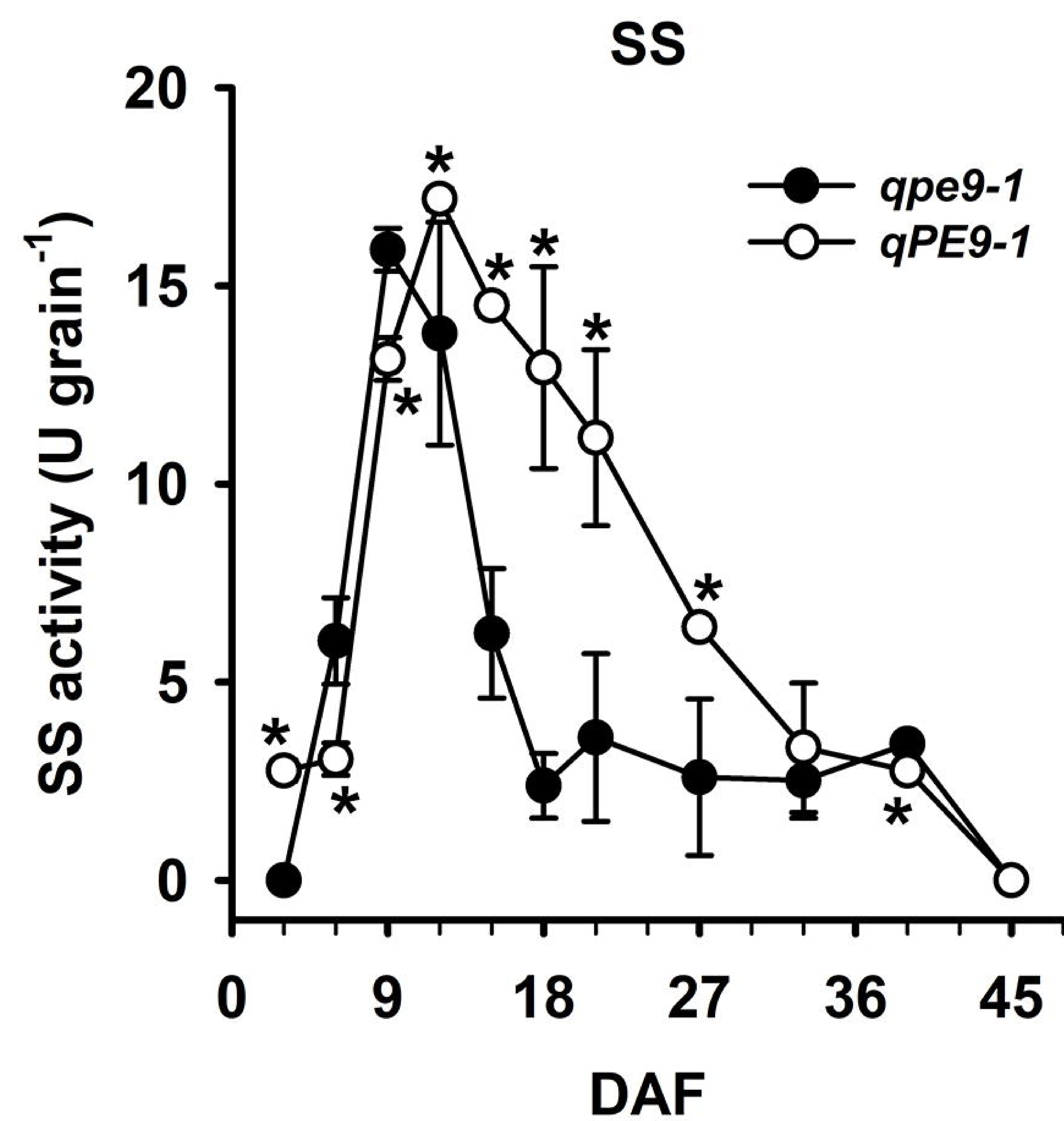

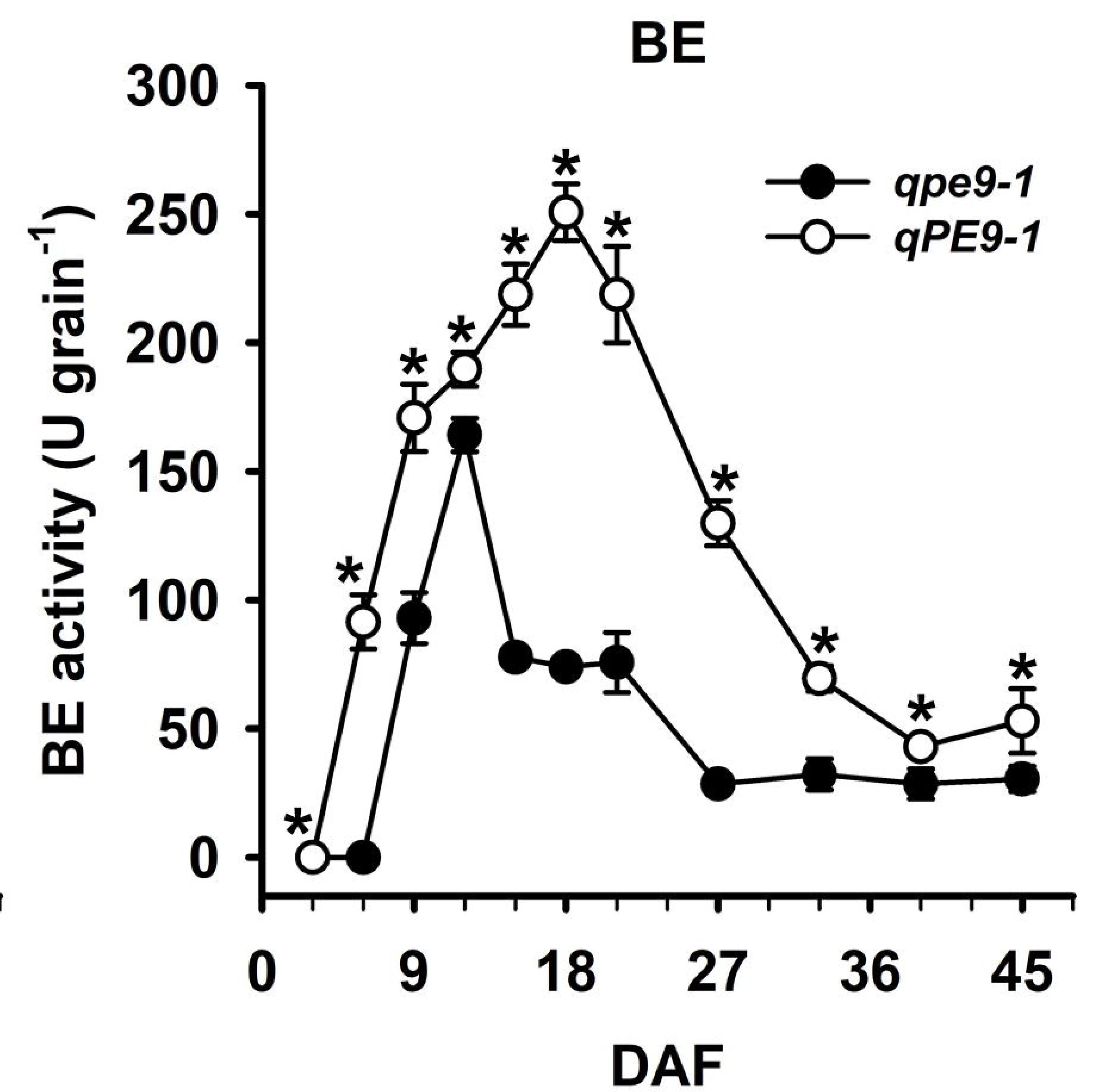

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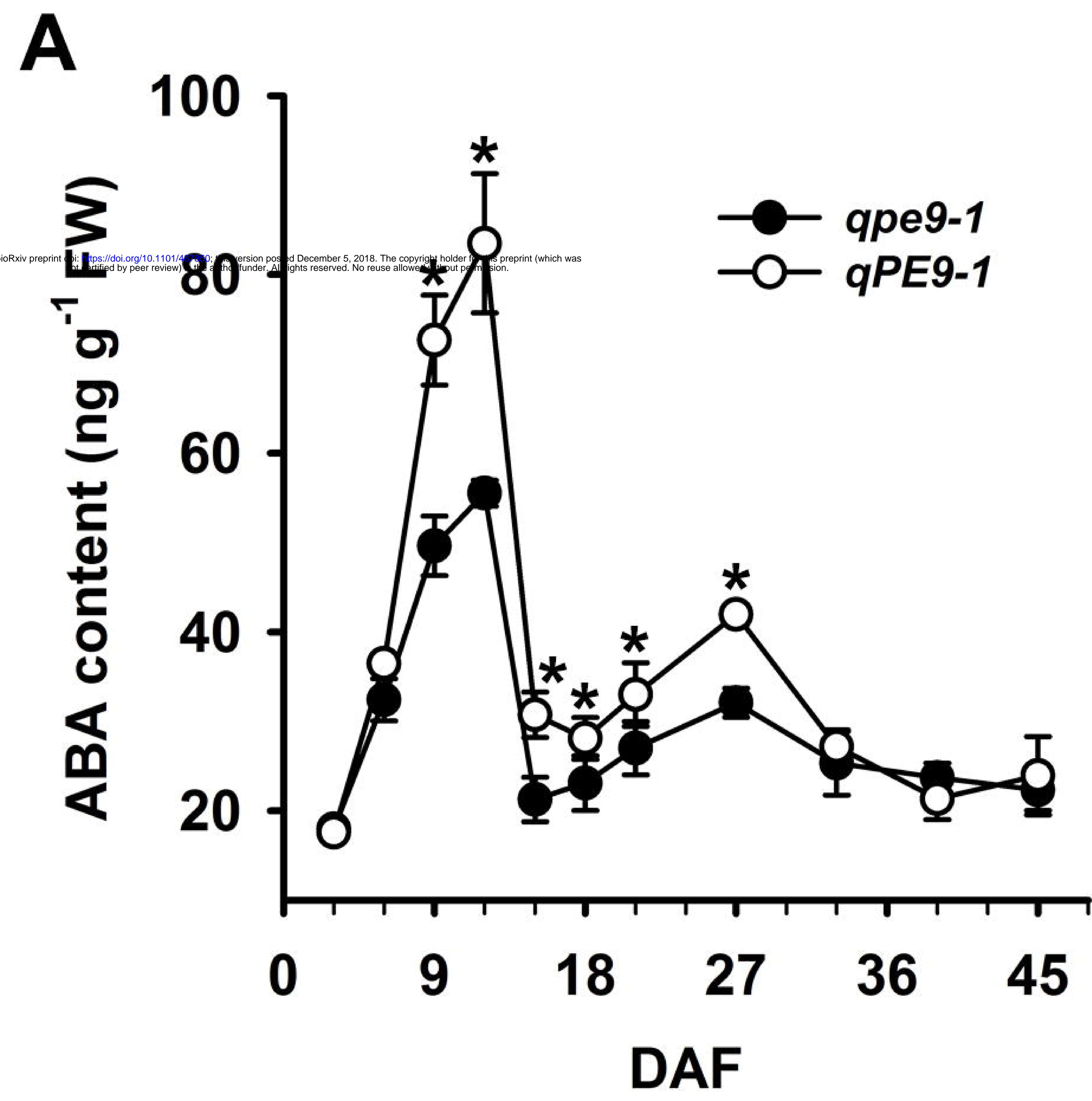

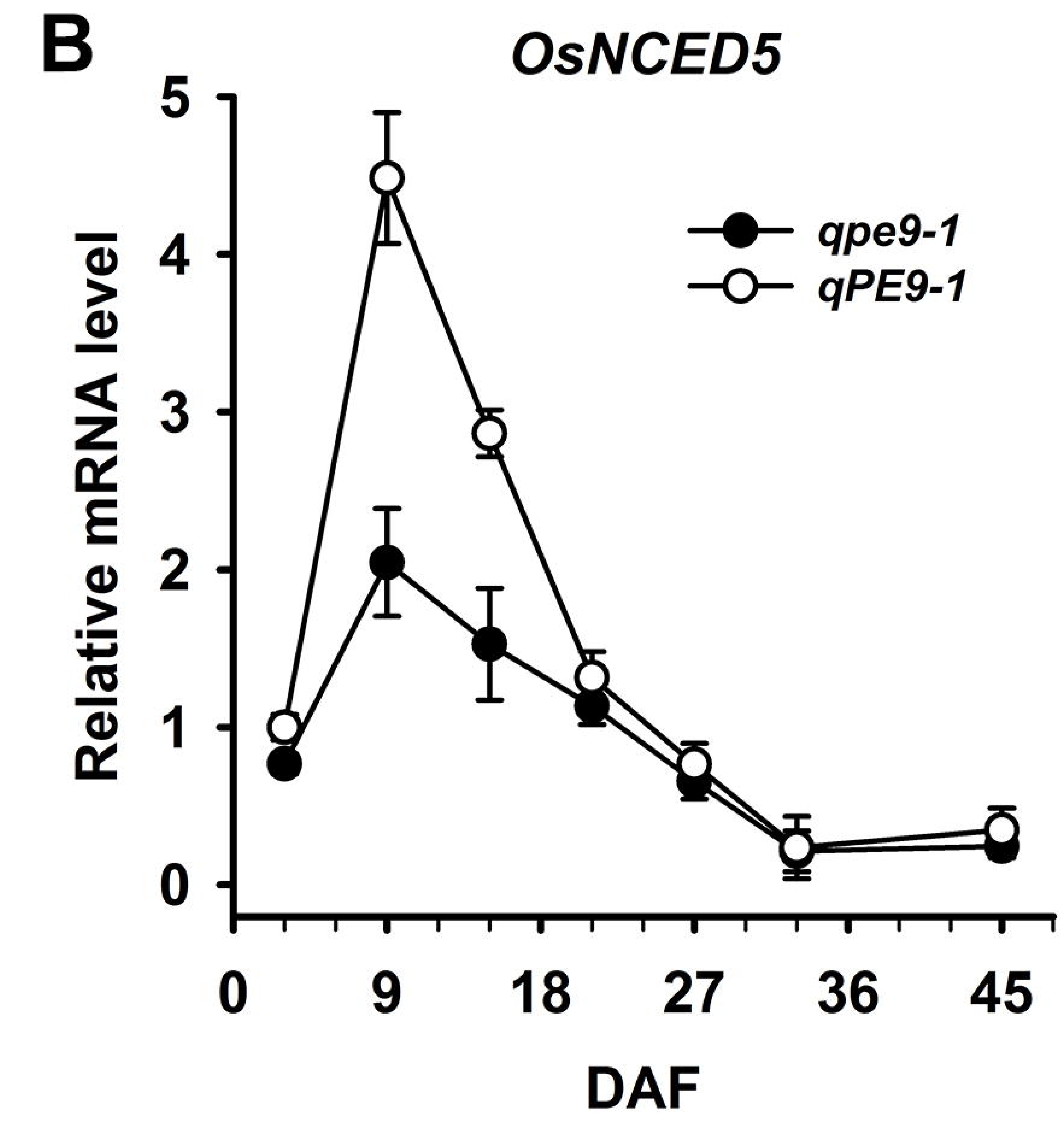

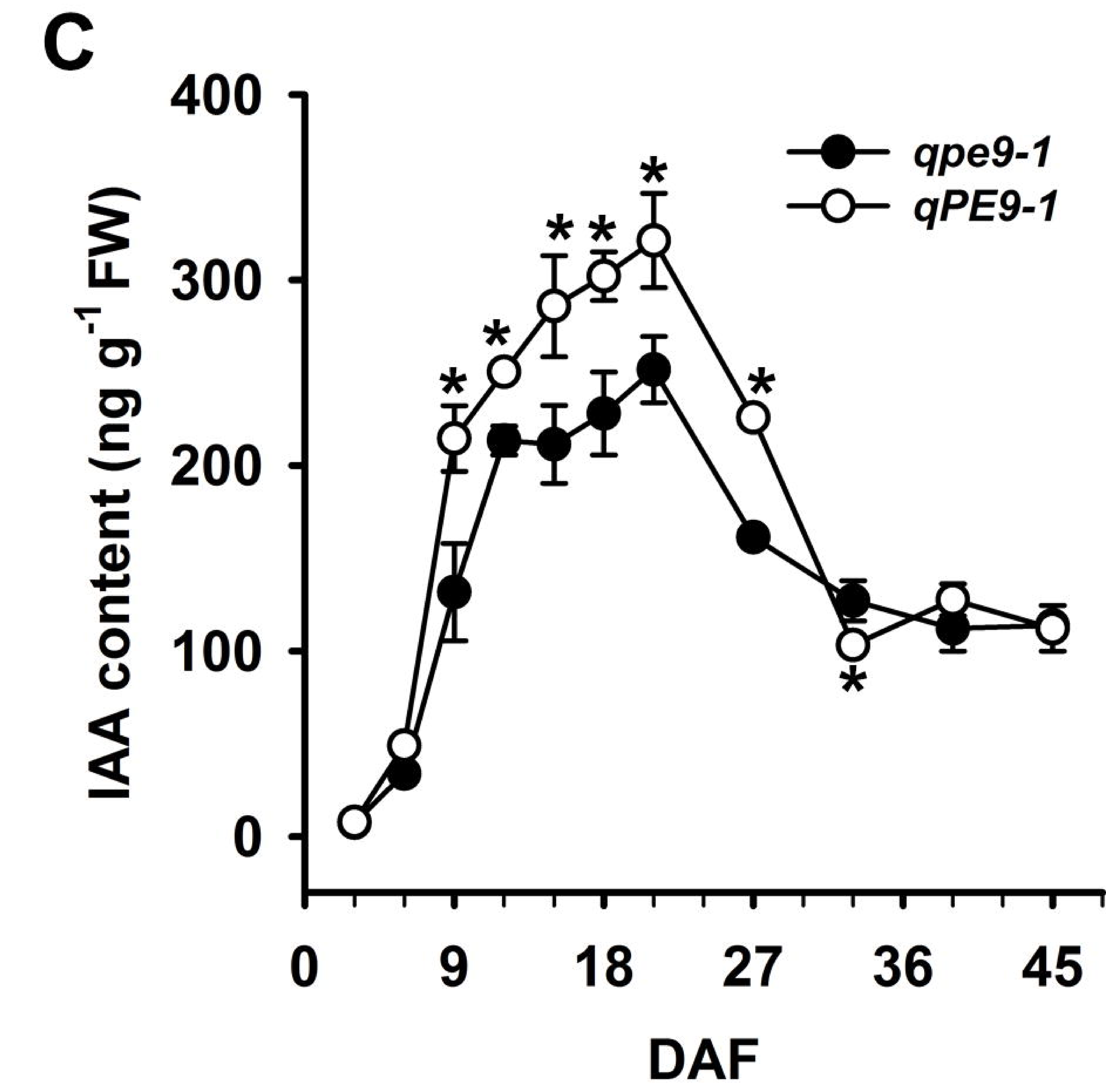

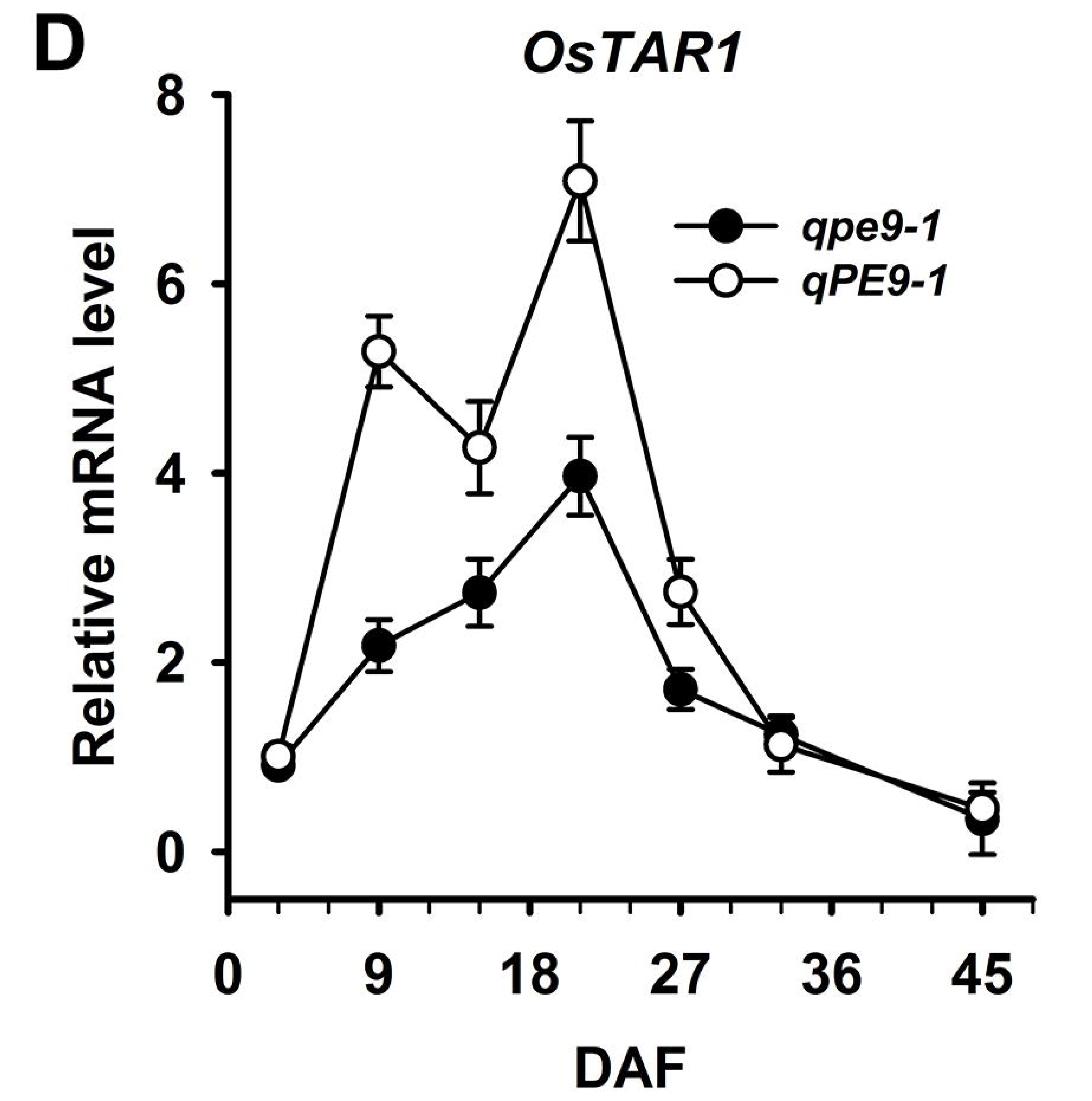

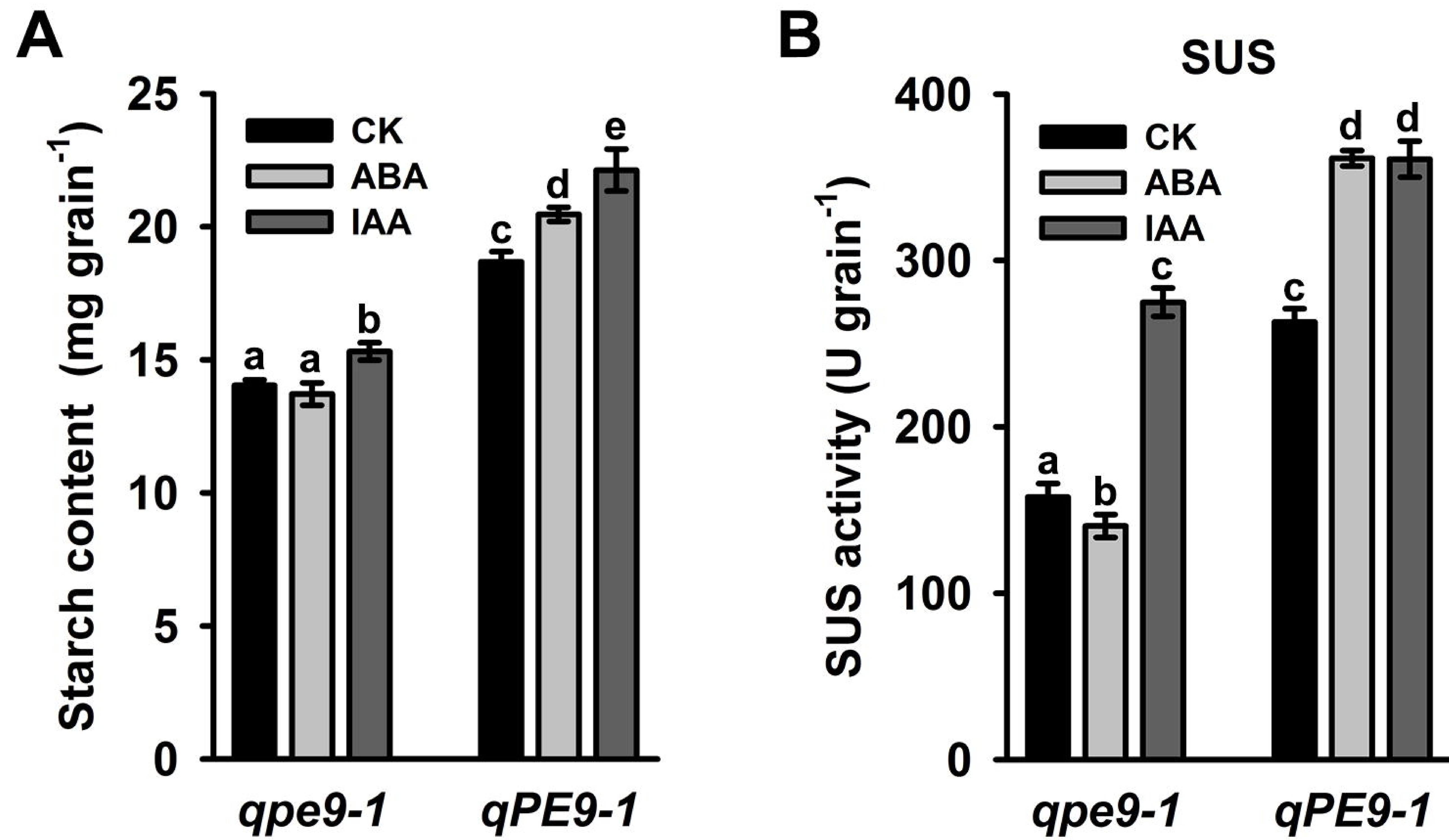

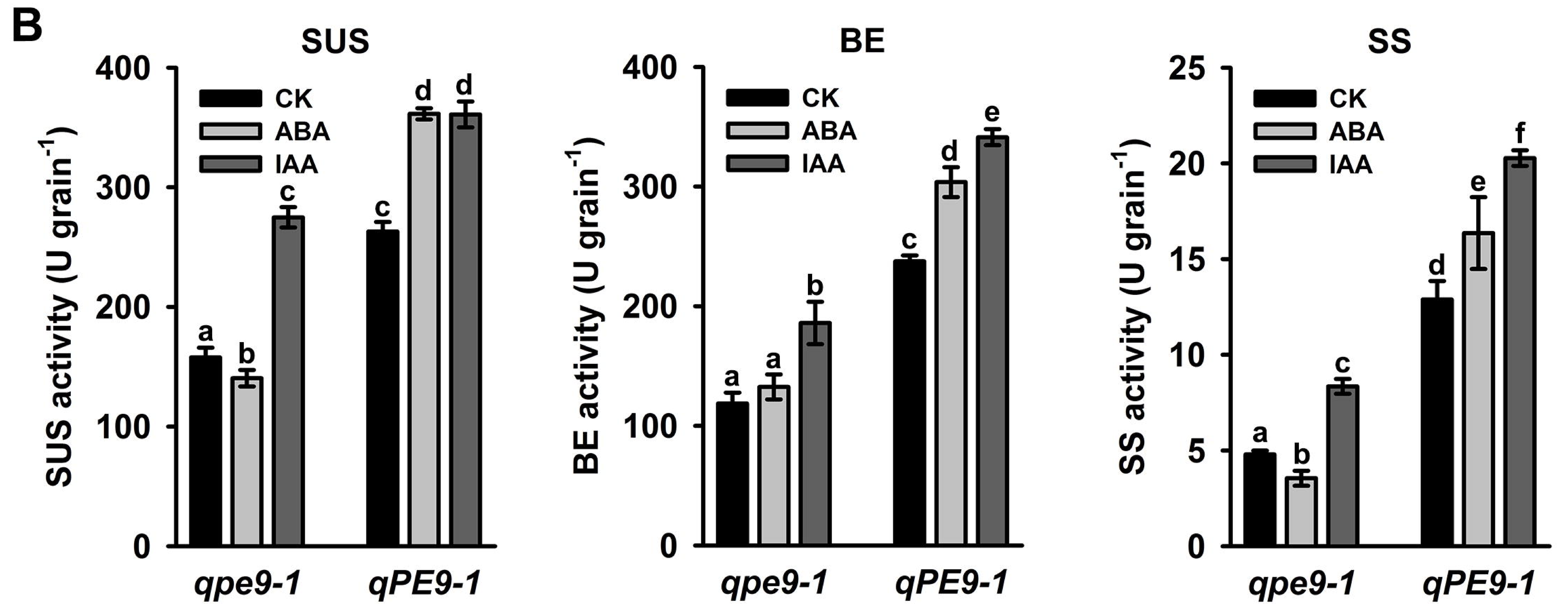

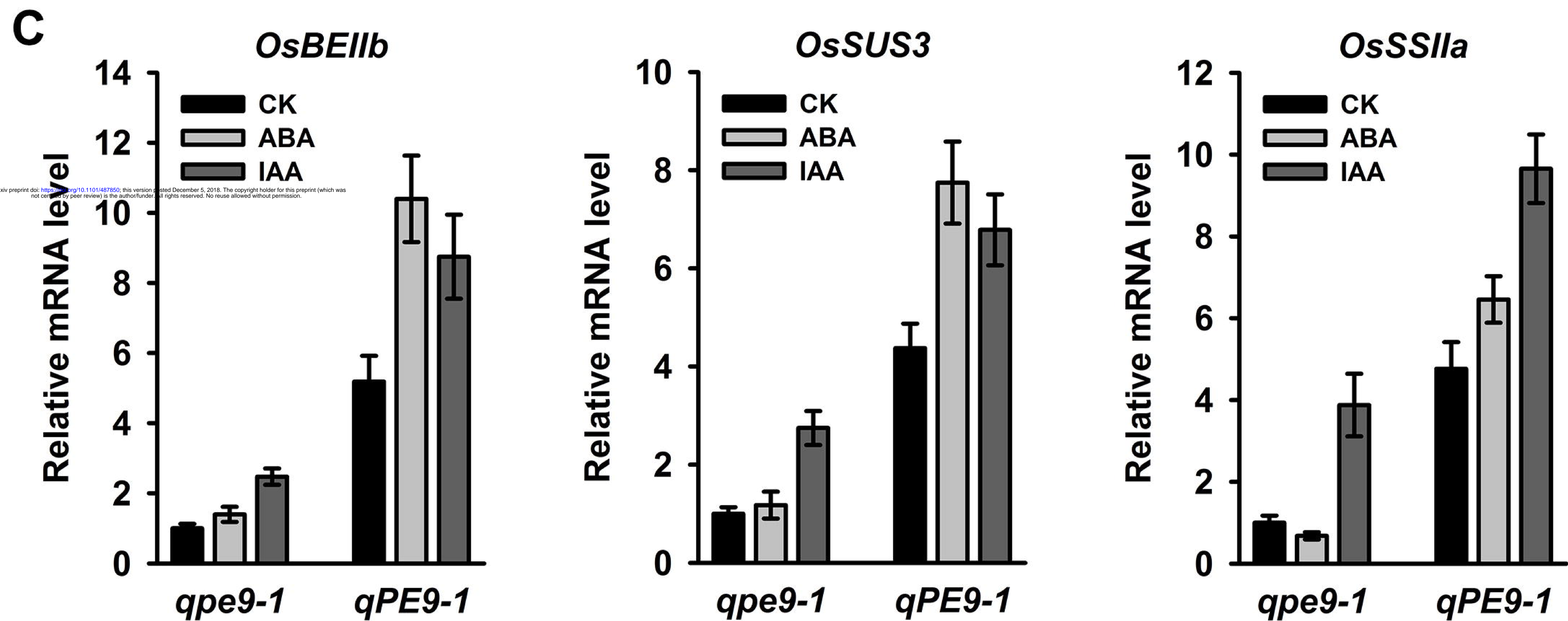

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