The Study of the Characteristics of the Secondary Flowering of *Cerasus subhirtella* 'Autumnalis'

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Abstract

The short flowering period of ornamental cherry trees is the main factor limiting their use in gardens. Determining the secondary flowering characteristics of ornamental cherry trees is required to prolong their flowering period. In this experiment, *Cerasus subhirtella* 'Autumnalis' was used as the experimental material. The phenological differences in their annual growth cycle were observed using the BBCH coding system. The cooling requirements of the flower buds were evaluated by the chilling hours model (temperature between 0 and 7.2 °C) and the Utah model. The expression of the core gene involved in bud dormancy regulation *DAM* (dormancy-associated MADS-box) from the completion of flower bud differentiation in one year until the following year was measured by performing real-time fluorescence-based quantitative PCR. The results showed that the flowering duration of *C. subhirtella* 'Autumnalis' from November to December was longer than that of *C. yedoensis* 'Somei Yoshino', which was from March to April. The progress from seed bud-break to flower bud opening took about 10 days for *C. subhirtella* 'Autumnalis', while the same stage for *C. yedoensis* 'Somei Yoshino' took around 20 days. Additionally, the flower buds of *C. subhirtella* 'Autumnalis' needed only the chilling

31 temperature unit of 54.08 to satisfy the chilling requirement, while *C. yedoensis* 'Somei Yoshino'

32 required a chilling temperature unit of 596.75. After the completion of flower bud differentiation,

33 during low-temperature storage, the expression of *DAM4* and *DAM5* genes first increased and

34 then decreased, whereas, the expression of the DAM6 gene continued to decrease, and the

35 expression of *DAM4*, *DAM5*, and *DAM6* in *C. yedoensis* 'Somei Yoshino' increased rapidly and

36 was maintained at a high level. This showed that the upregulation of the expression of the

37 *DAM4*, *DAM5*, and *DAM6* genes can inhibit the flower bud germination of Cherry Blossom. The

38 relative expression of the *DAM* gene of *C. subhirtella* 'Autumnalis' was significantly lower than

39 that of the *DAM* gene of *C. yedoensis* 'Somei Yoshino' from the end of October to the beginning

40 of December, leading to its secondary flowering in autumn. These results might elucidate why

41 the flower buds of *C. subhirtella* 'Autumnalis' can break their internal dormancy and bloom in

42 the autumn and then again in the following year. Our findings might provide a reference for

43 conducting further studies on the mechanisms of secondary flowering and bud dormancy in

44 cherries.

45

46 **Keywords**

47 Cherry blossom; Secondary flowering; Plant phenology; Chilling requirement; The DAM gene

48

49 **Introduction**

50   Cherry blossoms (*Cerasus* sp.) belong to the subfamily Prunus. of the Rosaceae family.

51 They belong to a flowering tree species with beautiful flowers and have a high ornamental value.

52 Cherry blossom is a widely used ornamental plant in urban green areas around the world, and it

53 generally flowers in April. The flowering period is relatively short, which restricts its wide

54 application as an ornamental plant in spring. Therefore, cultivating new cherry varieties with an

55 improved flowering period is required. The cherry variety *C. subhirtella* 'Autumnalis' flowers in

56 spring and October, which gives rise to a secondary flowering period. This flowering

57 characteristic is very stable. Utilizing this secondary flowering variety can further develop the

58 ornamental value of flowers in autumn, expand the effective ornamental period of cherry, and

59 influence the cultivation of new cherry varieties with a favorable flowering period.

60   Many factors affect the stable secondary flowering of plants. The stages of flower bud

61 development differ extensively based on the species and the season. For example, studies on

62 flower bud differentiation of lilac (*Syringa oblata*) varieties with secondary flowering showed

63 that the flower buds of the same plant might have two mechanisms of flower bud differentiation,

64 which include the summer-autumn differentiation type and the primary differentiation type, in

65 the same year (Wang & Wang, 2009). The cooling requirement is an important index to measure

66 flower bud differentiation. During the dormancy period, the cooling requirements are not met,

67 and the plants cannot pass through dormancy smoothly. This causes many problems that are not

68 conducive to plant growth and development (Alburquerque et al., 2008; Liu et al., 2017; Wang et

69 al., 2003). A study on the mechanism of secondary flowering in tree peonies (*Paeonia*

70 *suffruticosa*) showed that most peony varieties with secondary flowering have low cooling

71 requirements for flower bud-break, shallow dormancy, and a short period of flower bud

72 expansion (Chen, 2000). Similar to peonies, cherry blossoms are plants that generally undergo

73 genetic differentiation during summer and autumn. The flower buds of these plants require

74 sufficient low-temperature storage at the dormant stage before they can germinate and open in

75 the following year. However, *C. subhirtella* 'Autumnalis' can open from November to December

76 of the same year after the flower bud differentiation is completed, indicating that the cooling

77 requirements and the dormancy process have changed.

78   Determining the mechanism of secondary flowering by assessing the molecular mechanism

79 of dormancy and dormancy release in plant buds and elucidating the endogenous process of

80 inducing buds to stop growth is important (Wang & Wang, 2009; Celgev & Su, 1955). The gene

81 related to dormancy in plants is called the *DAM* (dormancy-associated MADS-box) gene, which

82 was first identified by Bielenberg et al. in the peach tree (**Prunus persica**) using forward genetics

83 and map-based cloning (Bielenberg et al., 2008). Further studies on the *DAM* gene have shown

84 that this gene can control plant dormancy and flower bud-break. For example, the bud-break of

85 *Arabidopsis thaliana* seeds overexpressing the *DAM6* gene in cherry (*Cerasus pseudocerasus*)

86 was strongly inhibited, and the heterologous expression of the *DAM1* and *DAM5* genes in cherry

87 caused abnormal flower development in transgenic *Arabidopsis thaliana*. Most DAM genes can

88 directly or indirectly regulate bud dormancy in different species and inhibit bud growth,

89 indicating that they are highly conserved (Mei et al., 2018; Wan & Liu, 1979). Some studies on

90 peaches found that the expression levels of *DAM5* and *DMA6* were negatively correlated with

91 flower bud-break, and the relative expression of these genes was upregulated in cold

92 environments in the autumn. The expression of *DAM5* and *DAM6* in cold-demanding plant

93   varieties was significantly higher than that in low-temperature cold-demanding varieties

94   (Yamane et al., 2011). Horvath et al. showed that the *DAM* homolog *EeDAM* might be involved

95   in the induction of endodormancy in the buds of leafy spurge (*Euphorbia esula* L.) (Jiang et al.,

96   2007). Jiménez et al. studied three peach tree varieties and found that the expression of *DAM*

97   genes was higher before low-temperature storage. Under field conditions, the GC content of

98   *DAM5* increased, followed by the decrease in the GC content of *DAM4* and *DAM6* genes after

99   storage at low temperatures. When the expression of these genes in all varieties reached the

100   lowest value, different varieties of peach trees had the same bud break capacity (Jiménez et al.,

101   2010). A study on pear flower bud dormancy elucidated a molecular regulation mechanism

102   involving the *DAM* gene. An increase in the relative expression of the *DAM* gene can induce

103   plant buds to enter internal dormancy and maintain the state of intrabud dormancy by inhibiting

104   the expression of the *FT2* gene (Chen, 2000). The *DAM* genes are most likely to be induced by

105   low-temperature storage and variations in the internal dormancy of *C. subhirtella* 'Autumnalis'.

106   The *DAM4*, *DAM5*, and *DAM6* genes are responsible for its secondary flowering. The expression

107   level of these genes is the key indicator to elucidate the mechanism of secondary flowering of *C.*

108   *subhirtella* 'Autumnalis'.

109   The secondary flowering phenology in plants has been recorded and reported across the

110   world. The double-flowered plants include apple, pear, cherry blossom, lilac, plum (*Armeniaca*

111   *mume*), Begonia (*Malus spectabilis*), Peony, etc. However, due to the lack of recognized

112   phenological observation indicators, the comparison of phenological characteristics between

113   different regions is hindered. The BBCH coding system (Biologische Bundesanstalt,

114   Bundessortenamt, and Chemische Industrie) has provided unified quantitative standards for

115   phenological observations and comparisons of plants in different regions and ways to solve the

116   problem of inconsistent information on plant phenological developmental stages. The BBCH

117   system is based on the Zadoks model and is jointly developed by BASF, Bayer, Ciba-Geigy, and

118   Hoechst. It is a decimal coding system for describing the growth and development of herbaceous

119   and woody plants and solves the problem of inconsistent description of plant phenological

120   development scales. It is recognized and used by many countries and international organizations

121   (Zadoks et al., 1974; Zhang et al., 2011).

122   In this experiment, the double-flowered cultivar *Cerasus subhirtella* 'Autumnalis' (2n = 3x =

123   24) was used as the experimental material, and the once-flowering cultivar *Cerasus yedoensis*

124 'Somei Yoshino' (2n = 2x = 16) was used as the control. The two Sakura cultivars had the same

125 parents. *Cerasus yedoensis* 'Somei Yoshino' is the most widely used and representative cherry

126 variety in gardens (Wang & Huang, 2001). By determining the differences in phenological

127 changes, cooling requirements, and DAM gene expression changes between the two varieties,

128 the mechanism of secondary flowering of *Cerasus subhirtella* 'Autumnalis' was elucidated. This

129 study was conducted to provide new insights into the flowering of the genus Sakura, information

130 on the bi-annual flowering of plants, and a theoretical basis for the related research on the

131 application of cherry blossom in gardens.

132

133 **Materials & Methods**

134 **Experimental Materials**

135 The experimental materials were collected from the campus of Qingdao Agricultural University

136 (120°39′E, 36°32′N). The area has a warm temperate monsoon climate, an annual average

137 temperature of 13.6 °C, a large daily temperature variation, and annual average precipitation of

138 467.4 mm. The cherry blossom varieties selected in the experiment were the double-flowered

139 cultivar *C. subhirtella* 'Autumnalis' and the single-flowered cultivar *C. yedoensis* 'Somei

140 Yoshino', which had the same parents. For each variety, three plants with similar growth rates

141 and sizes were selected for the experiment.

142

143 **Experimental Method**

144 Phenological Observation

145 From June 2019 to April 2021, the phenological observations and recordings were made based

146 on the date of appearance of *C. subhirtella* 'Autumnalis' and *C. yedoensis* 'Somei Yoshino'.

147 Observations were made every 3–5 days. When the phenological periods of these plant species

148 overlapped and the plants grew rapidly, observations were made every day. We stopped

149 monitoring the plants after they stopped growing in the winter. The specific phenological

150 observation methods were developed based on the methods adopted by Wan Minwei and Fadón.

151 (Fadón et al., 2015; Wan & Liu, 1979). The timing of the growth and plant phenology of each

152 plant was recorded using the plant BBCH coding system. In this study, based on the BBCH

153 coding system, the coding method for the secondary flowering of cherry blossoms was

154 developed with repetitive phenological events in plants.

155

156   Assessment of the Chilling Requirement

157   The branches of *C. subhirtella* 'Autumnalis' and *C. yedoensis* 'Somei Yoshino' were collected in

158   mid-October 2019. Each sampling set contained five long branches ($\geq$ 15 cm) and five short

159   branches ($\leq$ 10 cm). The length and width of the buds on the branches were recorded every six

160   days. The collected branches were maintained in an artificial climate room for subsequent

161   cultivation. The artificial climate room was maintained at 24 °C/18 °C (day/night) and 62%

162   relative humidity. The bottom of the branches was cut off obliquely. The water was changed

163   every two days, and the base of the branches was cut 3~4 mm to ensure complete water uptake.

164   The data on field temperature and humidity were recorded with the COS-03 automatic

165   temperature and humidity recorder, and the instrument was set to record data once every 10 min.

166   The bud-break data was statistically analyzed based on the method developed by Wanglirong et

167   al. (Wang et al., 2003). For the bud-break rate, the grading of flower bud-break status was as

168   follows: Level 1, no bud-break; Level 2, budding; Grade 3, bud tip exposed green; Grade 4,

169   showing petals; Level 5, flowers open. The bud-break rate is the weighted average value of

170   flower buds at all levels, and the weight is the stage of the flower bud-break state. The grading of

171   the chilling requirement was as follows: a bud-break rate $\geq$ 2.5 indicated that the low temperature

172   was passed; for 2.5 < bud-break rate $\leq$ 3.0, the cooling demand was calculated based on the

173   accumulated low-temperature hours in the field in this sampling period; for 3.0 < germination

174   rate $\leq$ 3.5, the chilling requirement was calculated based on the arithmetic mean of the field

175   accumulated low-temperature hours in this period and the last sampling period; for bud-break

176   rate >3.5, the chilling requirement of the last sampling period was considered to prevail. The 0 ~

177   7.2 °C model and the Utah model were used as the estimation model of the chilling requirement,

178   and the formula to calculate the chilling requirement is as follows:

179 $$CH = \sum_{i=1}^{t} T, \quad T = \begin{cases} 1 (0\,℃ \leq T \leq 7.2\,℃) \\ 0 (else) \end{cases}$$

180
$$Utah = \sum_{i=1}^{t} T_u, T_u = \begin{cases} T \leq 1.4 \quad \square\square 0 \\ 1.4 \ \square \ < T \leq 2.4 \ \square\square 0.5 \\ 2.4 \ \square \ < T \leq 9.1 \ \square\square 1 \\ 9.1 \ \square \ < T \leq 12.4 \ \square\square 0.5 \\ 12.4 \ \square \ < T \leq 15.9 \ \square\square 0 \\ 15.9 \ \square \ < T \leq 18.0 \ \square\square \text{-}0.5 \\ T \geq 18.0 \ \square\square \text{-}1 \end{cases}$$

181 ### Extraction and Identification of the DAM Gene

182 Sampling was performed in mid-September 2020 when the flower bud differentiation of *C.*

183 *subhirtella* 'Autumnalis' and *C. yedoensis* 'Somei Yoshino' was almost completed. Flower buds

184 were collected every 15 days, 11 samples in total, and the branches that extended toward the sun

185 were selected. The collected branches were placed in an ice box and transported to the

186 laboratory. The collected flower buds were wrapped in tin foil, quickly frozen with liquid

187 nitrogen, and stored in a refrigerator at −80 °C until analysis. The genes related to cooling

188 requirements were searched in the NCBI (National Center for Biotechnology Information)

189 database, and real-time quantitative PCR primers were designed based on the gene sequences

190 reported in other studies (Qiu et al., 2020) (Chen, 2017). The names of the synthetic primers and

191 the upstream and downstream sequences used are presented in Table 1.

192   The total RNA of the samples was extracted using the Tiangen Polysaccharide Polyphenol

193 Plant Total RNA extraction kit (DP441), and the obtained RNA solution was stored in a

194 refrigerator at −80 °C to prevent degradation. The quality of the extracted RNA was visually

195 assessed by agarose gel electrophoresis, and *cDNA* was synthesized using the PrimeScript™ 1st

196 Strand *cDNA* Synthesis Kit (TaKaRa Bio Inc.). RSP3 was used as the internal reference gene in

197 the real-time quantitative PCR (qPCR) technique, and the relative expression of the *DAM4*,

198 *DAM5*, and *DAM6* genes in cherry blossoms was determined using the MA-6000 real-time

199 quantitative PCR system.

200

201 **Data Processing**

202 The qPCR data were analyzed using the StepOne Software and then exported to Microsoft Excel

203 and sorted into tables. The $2^{-\Delta\Delta Ct}$ method was used as a relative quantification strategy for the

204 quantitative analysis of the data. All charts were made using Microsoft Excel.

205

Results

Phenological Observations

208 At the Qingdao Agricultural University, from June 2019 to April 2021, the annual growth and

209 development cycles of *C. subhirtella* 'Autumnalis' and *C. yedoensis* 'Somei Yoshino' were

210 observed, and the results of the phenological observations of two varieties of cherry blossoms

211 were obtained. The phenological observations covered the whole growing season within the year.

212 The main period of the development of *C. subhirtella* 'Autumnalis' and *C. yedoensis* 'Somei

213 Yoshino' ranges from the bud development until all leaves are dropped, covering 6 of the 10

214 main growth stages based on the BBCH-scale system, including main growth stage 0: bud

215 development, main growth stage 1: leaf development, main growth stage 3: shoot development,

216 main growth stage 5: reproductive development, main growth stage 6: flower development, and

217 main growth stage 9: defoliation initiation and dormancy of trees. The timing of the phenological

218 stages is shown in Table 2. The expression method of the secondary flowering phenology of *C.*

219 *subhirtella* 'Autumnalis' was developed by using the normal BBCH coding method for the first

220 flowering (autumn flowering period) and adding the parentheses after the two-digit BBCH code

221 of the first flowering to describe the second flowering (spring flowering period). Arabic

222 numerals from 0 to 9 within the parentheses were then used to indicate the second repeat and

223 complete flowering phenology.

224   After the flower buds sprouted in spring, the leaf buds of both the cherry blossom varieties

225 started expanding in mid-March, and most leaves developed. These plants first bloomed and then

226 developed leaves. Some leaves expanded fully from the end of March to the beginning of April.

227 Most leaves unfolded fully in mid-April and late April, and the development lasted for about 30

228 days. The shoot development took longer, from the beginning of April to the end of August

229 (about 140 days). Different branches of the same plant were in different stages of development,

230 and the shoots at the top of the crown required the longest time to develop. A few leaves of *C.*

231 *yedoensis* 'Somei Yoshino' started changing color at the end of September, while those of *C.*

232 *subhirtella* 'Autumnalis' changed color at the beginning of October. Both varieties started

233 shedding leaves in mid-October, and all leaves were shed at the end of October. In deciduous

234 plants, the dropping of leaves lasts for about 40 days, and plant dormancy is established during

235 this time (Westwood, 2009).

236 After the differentiation of the flower buds of *C. yedoensis* 'Somei Yoshino' was completed,

237 the plant entered internal dormancy. The flower buds started germinating in early March and

238 developed in late March. The flower bud-break until the opening of the first bud took about 23

239 days. After the flower bud differentiation and the dormancy period of *C. subhirtella* 'Autumnalis'

240 were completed, the plant quickly broke its inner dormancy in October, and the flower buds

241 started germinating in early November. The ungerminated flower buds were forced into

242 dormancy and started germinating in mid-March of the following year. The period from the bud-

243 break of flower buds to the beginning of flowering took about 10 days. The required hours and

244 suitable conditions were created for flower buds to completely open in the fall. *Cerasus*

245 *subhirtella* 'Autumnalis' blooms twice a year. The first flowering occurs in autumn, while the

246 second flowering occurs in spring,the duration of the two flowering periods is shown in Figure

247 1,. After the flower buds germinate and open in autumn, the pedicels, sepals, and stamens remain

248 on the branches, until the bud breaks and opens in spring of the following year. The flower buds

249 fall off at about the same time,the whole flowering process is shown in Figure 2.

250

251 **The Chilling Requirement of Flower Buds**

252 The results(Table 3) of the Utah model to calculate the chilling requirement showed that the

253 minimum low-temperature storage of the flower buds of *C. subhirtella* 'Autumnalis' was 54.08

254 C·U, and the minimum chilling requirement of the flower buds of *C. yedoensis* 'Somei Yoshino'

255 was 596.75 C·U. The results of the 0–7.2 °C model to calculate the chilling requirement showed

256 that the flower buds of *C. subhirtella* 'Autumnalis' germinated without low-temperature storage,

257 and the lowest chilling requirement of the flower buds of *C. yedoensis* 'Somei Yoshino' was

258 523.00 C·H. Darbyshire et al. divided the cooling requirements of sweet cherries into four

259 intervals based on the level of cooling requirement, and the chilling requirement of the flower

260 buds of *C. subhirtella* 'Autumnalis' was lower than the standard with the minimum intervals of

261 chilling (Darbyshire et al., 2011). Overall, the two chilling requirement estimation models

262 showed that the chilling requirement for the flower bud-break of *C. subhirtella* 'Autumnalis' was

263 significantly lower than that of *C. yedoensis* 'Somei Yoshino'.

264

265 **Expression of the *DAM* Gene**

266 Changes in the Relative Expression of *DAM4*

267 The *DAM* gene triggers dormancy and inhibits flower bud-break in plants. In this study, after the
268 plant entered dormancy, the expression of the *DAM* gene was upregulated rapidly, and then, the
269 expression of the *DAM* gene was downregulated with storage at a low temperature. The relative
270 expression of the *DAM4* gene firstly increased and then decreased at the dormant stage of *C.*
271 *subhirtella* 'Autumnalis' and *C. yedoensis* 'Somei Yoshino' (Fig. 3), but the timing and amplitude
272 of the relative expression of the *DAM4* gene in these two varieties differed significantly.
273 Moreover, the relative expression of the *DAM4* gene in the flower buds of *C. yedoensis* 'Somei
274 Yoshino' and *C. subhirtella* 'Autumnalis' was extremely low in September. After the flower bud
275 differentiation was completed in mid-October, the relative expression of the *DAM4* gene in the
276 flower buds of *C. yedoensis* 'Somei Yoshino' increased till December. The relative expression of
277 *DAM4* reached the highest value in mid-December. In early January of the following year, the
278 flower buds of *C. yedoensis* 'Somei Yoshino' accumulated cold energy. The relative expression
279 of the *DAM4* gene decreased rapidly to the same level as that before entering dormancy by the
280 end of January. The relative expression of the *DAM4* gene in the flower buds of *C. subhirtella*
281 'Autumnalis', however, increased slightly in mid-October and decreased to the same level as that
282 before entering inner dormancy during autumn-flowering. The relative expression of the *DAM4*
283 gene was significantly upregulated in mid-December at the end of the autumn-flowering period.
284 In *C. subhirtella* 'Autumnalis', the upregulation occurred within a shorter period and at a lower
285 amplitude than that in *C. yedoensis* 'Somei Yoshino'.
286
287 Changes in the Relative Expression of *DAM5*
288 The functional analysis of MADS-box genes related to flower bud dormancy in the Chinese
289 cherry in other studies showed that the *DAM4*, *DAM5*, and *DAM6* genes might be involved in
290 flower bud dormancy and dormancy release (Shao, 2016). The relative expression of the *DAM5*
291 gene in the flower buds of *C. yedoensis* 'Somei Yoshino' first increased and then decreased
292 during dormancy (Fig. 4). However, the relative expression of the *DAM5* gene in the flower buds
293 of *C. subhirtella* 'Autumnalis' showed a fluctuating trend, i.e., it was first upregulated and then
294 downregulated, followed by the same trend. After the differentiation of the flower bud of *C.*
295 *yedoensis* 'Somei Yoshino' was completed in mid-September, the relative expression of the
296 *DAM5* gene increased rapidly, and in *C. subhirtella* 'Autumnalis', it increased at a lower rate
297 compared to that in *C. yedoensis* 'Somei Yoshino' in the same period. From October to

298 November, the relative expression of *DAM5* in *C. yedoensis* 'Somei Yoshino' was high, while in

299 *C. subhirtella* 'Autumnalis', it was first upregulated and then downregulated. The low-

300 temperature storage caused the inner dormancy to break, and the relative expression of *DAM5*

301 was the lowest during the autumn-flowering period. In mid-December, when *C. subhirtella*

302 'Autumnalis' entered dormancy, the relative expression of the *DAM5* gene in flower buds

303 increased, and the ungerminated flower buds could not continue to germinate. From mid-

304 December to early January, the relative expression of the *DAM5* gene in the flower buds of *C.*

305 *subhirtella* 'Autumnalis' was maintained at a high level, and then, it decreased till the end of

306 January. However, in the flower buds of *C. yedoensis* 'Somei Yoshino', the relative expression of

307 the *DAM5* gene remained at a high level from mid-December to early January. It decreased

308 gradually to the lowest level at this time, and the flower buds of *C. yedoensis* 'Somei Yoshino'

309 germinated and opened. These plants generally could not open because the external

310 environmental conditions were not suitable for their growth. At the end of February, when the

311 flower buds began germinating, the relative expression of *DAM5* in *C. yedoensis* 'Somei

312 Yoshino' and *C. subhirtella* 'Autumnalis' reached the lowest levels.

313

314 Changes in the Relative Expression of *DAM6*

315 The relative expression of the *DAM6* gene in the flower buds of *C. yedoensis* 'Somei Yoshino'

316 was first upregulated and then downregulated during dormancy (Fig. 5). It first decreased, then

317 increased, and finally, decreased again. During low-temperature storage, the relative expression

318 of the *DAM6* gene was rapidly downregulated and was the lowest during the autumn-flowering

319 period of *C. subhirtella* 'Autumnalis'; however, it increased after the autumn-flowering period

320 was over. In contrast, in the flower buds of *C. yedoensis* 'Somei Yoshino', the relative expression

321 of the *DAM6* gene remained at a high level during the same period.

322

323 **Discussion**

324 **Differences in Phenological Stages**

325 In Qingdao, the autumn-flowering period of *C. subhirtella* 'Autumnalis' is from early November

326 to mid-December, with a flowering duration of about 35 days, whereas, the spring-flowering

327 period is from the end of March to mid-April, with a flowering duration of about 20 days. The

328 spring-flowering period of *C. yedoensis* 'Somei Yoshino' is from mid-late March to mid-April,

329 and the flowering duration is about 16 days. The spring-flowering period of *C. subhirtella*

330 'Autumnalis' starts slightly earlier than that of *C. yedoensis* 'Somei Yoshino', and the duration of

331 the spring-flowering period of *C. yedoensis* 'Somei Yoshino' is slightly shorter than that of *C.*

332 *subhirtella* 'Autumnalis', but the flowering is more concentrated. The flowers of *C. yedoensis*

333 'Somei Yoshino' are in full bloom, and they have more flowers than *C. subhirtella* 'Autumnalis'.

334 By comparing the two flowering periods of *C. subhirtella* 'Autumnalis' within one year, we

335 found that the autumn-flowering period lasts longer than the spring-flowering period in this

336 variety.

337

338 **The Chilling Requirement of *C. subhirtella* 'Autumnalis' was Significantly Lower**

339 **than That of *C. yedoensis* 'Somei Yoshino'**

340 *Cerasus subhirtella* 'Autumnalis' can bloom in autumn, but the inconsistent flowering is closely

341 related to the lower chilling requirement and the characteristics of flower bud differentiation. The

342 time of initiation and the rate of flower bud differentiation in the same *C. subhirtella*

343 'Autumnalis' plant differ (Zheng, 2000). The low-temperature storage first occurs in those

344 flower buds that first complete the differentiation process. Due to a significant decrease in

345 chilling hours, the flower buds can gradually complete the low-temperature cycle in autumn.

346 Accumulation, breaking internal dormancy, bud-break, and opening occur before the temperature

347 drop triggers the flower buds to enter forced dormancy, which causes *C. subhirtella*

348 'Autumnalis' to bloom in autumn. However, flowering is not concentrated, and only a few

349 flowers occur in full bloom. The physiological mechanism of autumn-flowering of *C. subhirtella*

350 'Autumnalis' is similar to that of peonies (Wang, 2007).

351

352 **Relative Expression of *DAM* Genes**

353 The relative expression of the *DAM4* gene in *C. yedoensis* 'Somei Yoshino' and 'Yueyueying'

354 was considerably higher than that of *DAM5* and *DAM6* at the dormant stage. These results were

355 similar to those found by Chen Xiaodan in a study on sweet cherries (Chen, 2017). The *DAM4*

356 gene plays a dominant role in the bud dormancy process of cherry blossoms. The results of the

357 experiments showed that the relative expression of the *DAM4* gene in the flower buds of *C.*

358 *subhirtella* 'Autumnalis' with low cooling requirements during dormancy was significantly

359 downregulated compared to the relative expression of the *DAM4* gene in the flower buds of *C.*

360 *yedoensis* 'Somei Yoshino'. These results were similar to those reported by Ubi et al. The results

361 of the study on the *DAM* genes in the dormant stage were similar. The high-chilling requirement

362 cultivars had a high level of *DAM* gene expression in the dormant stage. With the release of

363 dormancy, the expression was downregulated, and the expression of the low-chilling requirement

364 cultivars also decreased significantly. During the autumn-flowering period, the relative

365 expression of the *DAM5* gene in *C. subhirtella* 'Autumnalis' was significantly lower than that in

366 *C. yedoensis* 'Somei Yoshino'. The *DAM5* gene inhibits the bud-break of flower buds. The bud-

367 break rate of buds was negatively correlated with the relative expression of the *DAM5* gene, and

368 the expression level in the flowers was extremely low, indicating that the *DAM5* gene could

369 inhibit bud-break. Another study (Balogh et al., 2019) also showed that *DAM5* and *DAM6* can

370 delay the dormancy release and flowering time and inhibit flowering in plants.

371 The *DAM6* gene may play an important regulatory role in the process of bud dormancy and

372 dormancy release in cherries. The relative expression of the *DAM6* gene first increased and then

373 decreased with low-temperature storage (Mei et al., 2018). Before entering the internal dormancy

374 period, the relative expression of *DAM6* in the flower buds of *C. yedoensis* 'Somei Yoshino' and

375 *C. subhirtella* 'Autumnalis' was higher. Different *DAM* genes can inhibit flower bud-break at

376 different times, and the *DAM6* gene mainly influences processes occurring before the completion

377 of flower bud differentiation.

378

Conclusions and Outlook

380 The flower bud differentiation of *C. subhirtella* 'Autumnalis' increased, and the period from

381 flower bud-break to flowering decreased, providing optimum conditions for buds to open in

382 autumn. The buds of *C. subhirtella* 'Autumnalis' had a lower cooling requirement, and the *DAM*

383 gene was expressed in the buds flowering in autumn. As low-temperature storage decreased

384 rapidly, flower buds could break their internal dormancy and continue to develop before entering

385 eco-dormancy, which is an important reason for the autumn-flowering of *C. subhirtella*

386 'Autumnalis'. Studies on the mechanism of the secondary flowering of *C. subhirtella*

387 'Autumnalis' are limited, and the experimental anatomical data on the structure of *C. subhirtella*

388 'Autumnalis' during the critical period of secondary flowering are insufficient. The mechanism

389 related to the effects of the *DAM4*, *DAM5*, and *DAM6* genes, and the regulatory mechanism of

390   the internal dormancy and release of internal dormancy in the flower buds of *C. subhirtella*

391   'Autumnalis' need further investigation.

392

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506   **Table 1.** Primer sequences

507   **Table 2.** The timing of phenological stages of two varieties of cherry blossom based on the

508   BBCH coding system

509   **Table 3.** The cooling requirements of *C. subhirtella* 'Autumnalis' and *C. yedoensis* 'Somei

510   Yoshino'

511   **Fig. 1.** Flower bud-break and the flowering duration of *C. subhirtella* 'Autumnalis' and *C.*

512   *yedoensis* 'Somei Yoshino'. Above the dotted line is *C. yedoensis* 'Somei Yoshino' and below

513   the dotted line is *C. subhirtella* 'Autumnalis'. (A) indicates the autumn-blooming period of

514   flower buds of *C. subhirtella* 'Autumnalis', (B) indicates the autumn-flowering period of *C.*

515   *subhirtella* 'Autumnalis', (C) indicates the spring-blooming period of *C. subhirtella*

516   'Autumnalis', (D) indicates the spring-flowering period of 'October Sakura', (E) indicates the

517   period of the flower bud expansion of *C. yedoensis* 'Somei Yoshino' in spring, and (F)

518   represents the spring-flowering period of *C. yedoensis* 'Somei Yoshino'.

519   **Fig. 2.** Flower bud-break and opening of *C. subhirtella* 'Autumnalis' and *C. yedoensis* 'Someiv'.

520   (I) indicates the process of flower bud-break and the opening of *C. subhirtella* 'Autumnalis' in

521   autumn, (II) indicates the process of flower bud-break and the opening of *C. subhirtella*

522   'Autumnalis' in spring, (III) indicates the process of flower bud-break and the opening of *C.*

523   *yedoensis* 'Somei Yoshino' in spring, (S1) indicates 51, the flower bud-swelling phase, (S2)

524   indicates 53, inflorescence enclosed by pale green scales, (S3) indicates 54, the pedicel begins to

525   elongate, (S4) indicates 55, the initial stage, (S5) indicates 57, the half-opening period, (S6)

526   indicates 59, the full-opening period.

527   **Fig. 3.** The relative expression of the DAM4 gene in the flower buds of *C. yedoensis* 'Somei

528   Yoshino' and *C. subhirtella* 'Autumnalis'.

529   **Fig. 4.** The relative expression of the DAM5 gene in the flower buds of *C. yedoensis* 'Somei

530   Yoshino' and *C. subhirtella* 'Autumnalis'.

531   **Fig. 5.** The relative expression of the DAM6 gene in the flower buds of *C. yedoensis* 'Somei

532   Yoshino' and *C. subhirtella* 'Autumnalis'.

533

Primer sequences

1 **Table 1** Primer sequences

Gene	Primer	Primer sequence	Purpose
RSP3	*RSP3-F*	TCAAGGTCAGGTAAGGGGGTC	Reference gene
	RSP3-R	GTGAGGTGATTGTTAGTGGAA	
DAM4	*DAM4-F*	ATCAGATGGTGATGTTAAAGG	full-length clone
	DAM4-R	TGGAGGTAGCAGATTCAGAT	
DAM5	*DAM5-F*	AATTGAATGATCAAGAGATTG	full-length clone
	DAM5-R	CAGCTCTTCCTTAGTTTCCATG	
DAM6	*DAM6-F*	GAGTGAGATCATGTCACTGGA	full-length clone
	DAM6-R	AGCTGGTAGAGGTGGCCATTG	

The timing of phenological stages of two varieties of cherry blossom based on the BBCH coding system

1 **Table 2** The timing of phenological stages of two varieties of cherry blossom based on the

2 BBCH coding system

Principal growth stage	BBCH Code	Description of secondary growth stage	*C. subhirtella* 'Autumnalis	*C. yedoensis* 'Someo-yoshino
0 shoot development	00	Leaf buds closed and covered with scales	03.09	03.12
	01	Leaf buds begin to swell and the buds have scales with light edges	03.12	03.14
	09	Green leaf tips visible	03.16	03.23
1 Leaf development	10	Green scales slightly open, leaves exposed	03.22	03.28
	11	Leaves unfold, developmental axis visible	03.25	04.01
	12	leaves fully expanded	04.03	04.07
3 Shoot development	31	Visible developmental bud axis	04.04	04.09
	32	Shoots 20% of the final length	04.16	04.20
	33	Shoots 30% of the final length	04.22	04.25
	35	Shoots 80% of the final length	07.20	07.15
	39	Shoots 90% of the final length	08.28	08.30
Flower development (autumn flowering period)	50	Buds start to swell	11.02	/
	53	Inflorescence surrounded by light green scales	11.06	/
	54	The pedicels start to elongate	11.08	/
	55	First bloom	11.12	/
	57	Half bloom	11.13	/
	59	Full bloom	11.13	/
5 flower development (spring flowering period)	50(0)	Dormant, inflorescence buds closed	03.01	02.27
	51(1)	Inflorescence buds swollen	03.10	03.02
	53(3)	Inflorescence surrounded by light green scales	03.16	03.12
	54(4)	The pedicels start to elongate	03.19	03.17
	55(5)	First bloom	03.20	03.22
	57(7)	Half bloom	03.21	03.23

	59(9)	Full bloom	03.22	03.23
Flower development (autumn flowering period)	60	Flowers bloom	11.12	
	61	Flowering begins	11.12	
	62	60% of flowers open	11.18	/
	64	90% of flowers open	11.24	/
	67	Flowers begin to withe	11.18	/
	69	End of florescence	12.14	/
6 flower development (spring flowering period)	60(0)	Flowers bloom	03.22	03.23
	61(1)	Flowering begins	03.24	03.23
	62(2)	20% flowers open	03.25	03.24
	64(4)	60% flowers open	03.27	03.25
	65(5)	90% flowers open	04.01	03.28
	67(7)	Flowers begin to withe	03.30	03.30
	69(9)	End of florescence	04.12	04.10
9 Leaves fall, dormancy begins	91	The twig growth is completed, and the leaves are still completely green	08.28	09.08
	92	Blade starts to change color	09.28	09.16
	93	Falling leaves begin	10.17	10.22
	95	50% of blades fall off	11.08	11.10
	97	All leaves fallen	11.28	11.24

The chilling requirements of *C. subhirtella* 'Autumnalis' and *C. yedoensis* 'Somei yoshino'

1 **Table 3** The chilling requirements of *C. subhirtella* 'Autumnalis' and *C. yedoensis* 'Somei

2 yoshino'

Variety	0~7.2 °C model		Utah model	
	Starting and ending time	Results of cooling requirement	Starting and ending time	Results of cooling requirement
C. subhirtella 'Autumnalis'	11/20-	0 C·H	10/22–11/7	54.08 C·U
C. yedoensis 'Someo-yoshino'	11/20–1/10	523.00 C·H	10/22–1/10	596.75 C·U

Figure 1

Fig. 1 Flower bud germination and the flowering duration of C. subhirtella 'Autumnalis' and C. yedoensis 'Somei yoshino'

(A) indicates the autumn-blooming period of flower buds of C. subhirtella 'Autumnalis', (B) indicates the autumn-flowering period of C. subhirtella 'Autumnalis', (C) indicates the spring-blooming period of C. subhirtella 'Autumnalis', (D) indicates the spring-flowering period of 'October Sakura', (E) indicates the period of the flower bud expansion of C. yedoensis 'Somei yoshino' in spring, (F) represents the spring-flowering period of C. yedoensis 'Somei yoshino'
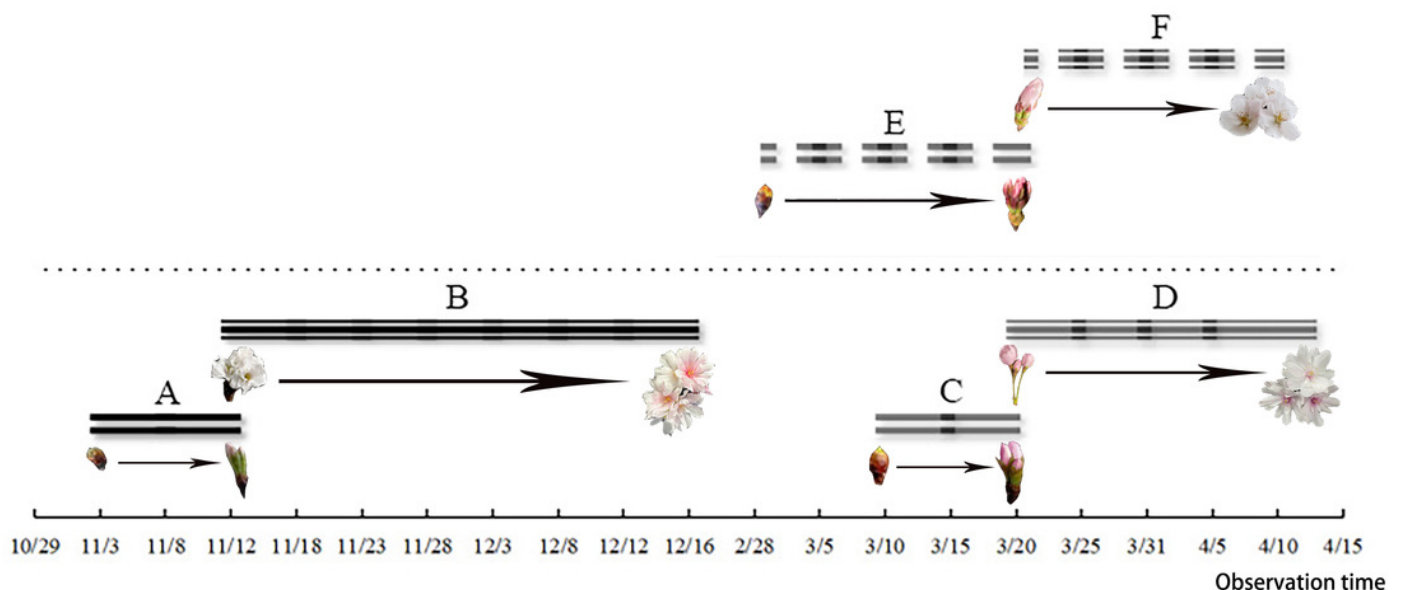
Figure 2

Flower bud germination and opening of C. subhirtella 'Autumnalis' and C. yedoensis 'Somei yoshino'

(I) indicates the process of flower bud germination and opening of C. subhirtella 'Autumnalis' in autumn, (II) indicates the process of flower bud germination and opening of C. subhirtella 'Autumnalis' in spring, (III) indicates the process of flower bud germination and opening of C. yedoensis 'Somei yoshino' in spring, (S1) indicates 51, The flower bud-swelling phase, (S2) indicates 53, Inflorescence enclosed by pale green scales, (S3) indicates 54, Pedicel begins to elongate, (S4) indicates 55, Initial stage, (S5) indicates 57, The half-opening period, (S6) indicates 59, The full-opening period

I

II

III

51 Flower buds
begin to swell

53 Inflorescence
surrounded by pale
green scales

54 Pedicel begins
to elongate

55 Initial stage

57 Half open period

59 Full opening period

10mm

Figure 3

Relative expression of the DAM4 gene in flower buds of C. yedoensis 'Somei yoshino' and C. subhirtella 'Autumnalis'
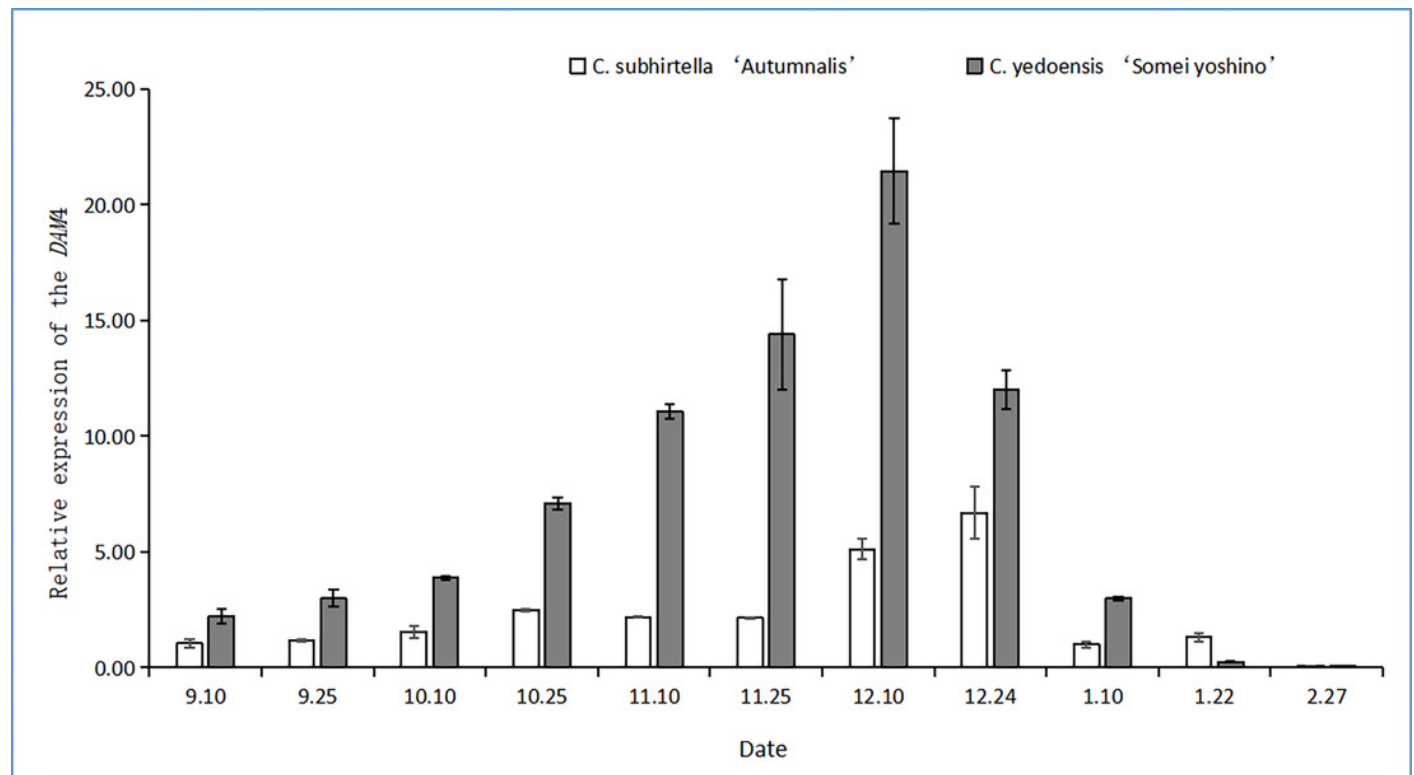
Figure 4

Relative expression of the DAM5 gene in flower buds of C. yedoensis 'Somei yoshino' and C. subhirtella 'Autumnalis'
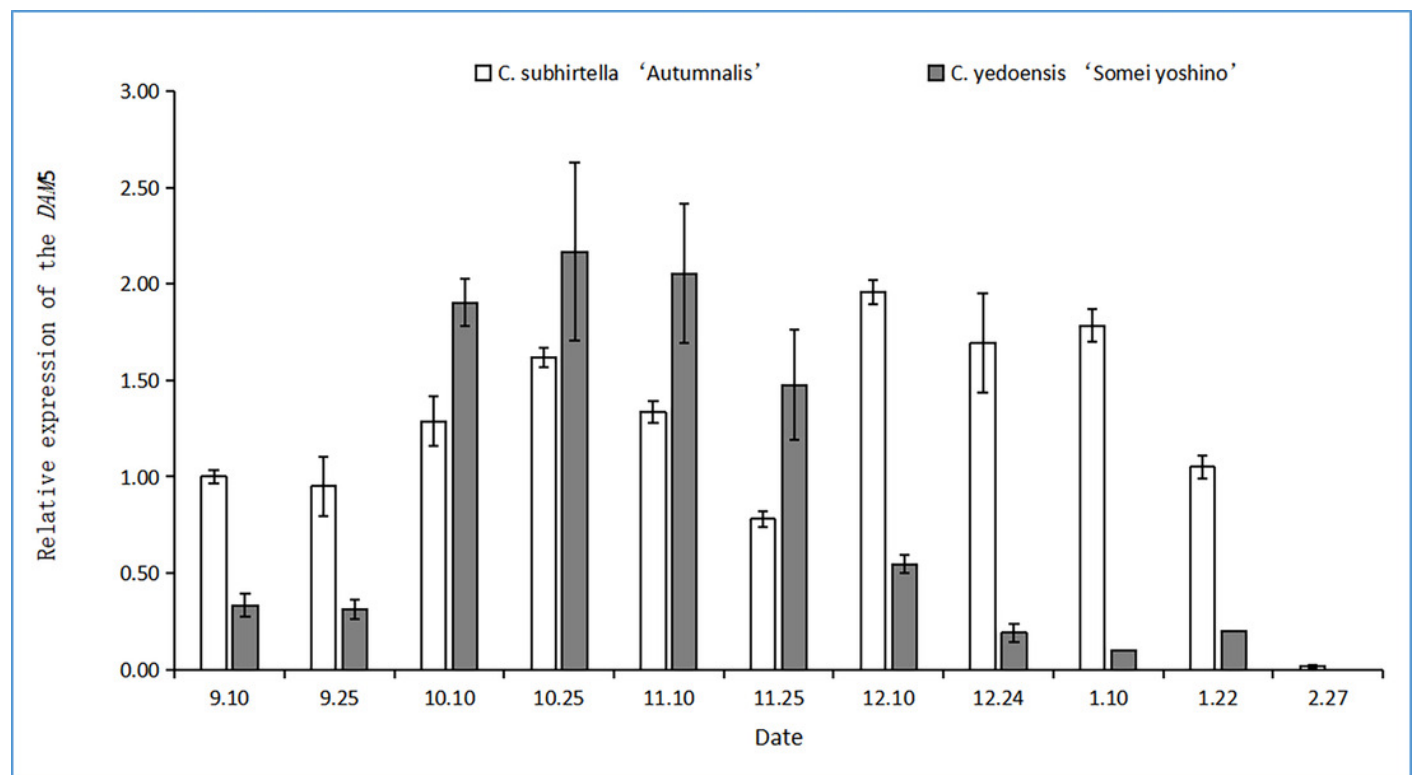
Figure 5

Relative expression of the DAM6 gene in flower buds of C. yedoensis 'Somei yoshino' and C. subhirtella 'Autumnalis'