10-1-2020

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Yongbo Duan
Huaibei Normal University

Franklin Eduardo Melo Santiago
Federal University of Lavras

Andre Rodrigues dos Reis
São Paulo State University

Marislaine A. de Figueiredo
Federal University of Lavras

Suping Zhou
Tennessee State University

See next page for additional authors

Recommended Citation

Y. Duan, F.E.M. Santiago, A.R. dos Reis, M.A. de Figueiredo, S. Zhou, T.W. Thannhauser, L. Li, "Genotypic variation of flavonols and antioxidant capacity in broccoli", Food Chemistry, Volume 338, 2021, 127997, ISSN 0308-8146, https://doi.org/10.1016/j.foodchem.2020.127997.

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Yongbo Duan[a,b], Franklin Eduardo Melo Santiago[a,c], Andre Rodrigues dos Reis[a,d], Marislaine A. de Figueiredo[a,e], Suping Zhou[f], Theodore W. Thannhauser[a], Li Li[a,g,*]

[a] *Robert W. Holley Center for Agriculture and Health, USDA-ARS, Cornell University, Ithaca, NY 14853, USA*

[b] *Key Laboratory of Resource Plant Biology of Anhui Province, College of Life Sciences, Huaibei Normal University, Huaibei, Anhui 235000, China*

[c] *Department of Soil Science, Federal University of Lavras, Zip Code 3037, Lavras, MG 37200-000, Brazil*

[d] *School of Science and Engineering, São Paulo State University (UNESP), Zip Code 17602-496, Tupã, SP, Brazil*

[e] *Department of Agriculture, Federal University of Lavras, Zip Code 3037, Lavras, MG 37200-000, Brazil*

[f] *Department of Agricultural and Environmental Sciences, Tennessee State University, Nashville, TN 37209-1561, USA*

[g] *Plant Breeding and Genetics Section, School of Integrative Plant Science, Cornell University, Ithaca, NY 14853, USA*

*** Corresponding author at:**

Robert W. Holley Center for Agriculture and Health, USDA-ARS

Plant Breeding and Genetics Section

School of Integrative Plant Science

Cornell University

Ithaca, NY 14853, USA

Phone: +1 607-255-5708

E-mail: ll37@cornell.edu

E-mail addresses of coauthors:

Yongbo Duan: yboduan@163.com

Franklin Eduardo Melo Santiago: franklin.santiago@hotmail.com.br

Andre Rodrigues dos Reis: andre.reis@unesp.br

Marislaine A. de Figueiredo: marislaine_alves@yahoo.com.br

Suping Zhou: ZSUPING@Tnstate.edu

Theodore W. Thannhauser: tt34@cornell.edu

Li Li: ll37@cornell.edu

ABSTRACT

Flavonols are gaining increasing interests due to their diverse health benefits for humans. Broccoli is a main flavonol source in our diet, but the genetic variation of flavonols and their correlation with antioxidant capacity remain to be understood. Here, we examined variations of the two major flavonols kaempferol and quercetin in florets and leaves of 15 diverse broccoli accessions by Ultra-Performance Liquid Chromatography (UPLC). Broccoli accumulated more kaempferol than quercetin in most of the accessions tested, with the ratios varying from 4.4 to 27.9 in leaves and 0.4 to 4.4 in florets. Total flavonoids showed 2.5-fold and 3.3-fold differences in leaves and florets of these accessions, respectively. Principle components analysis revealed that flavonols, along with key biosynthetic genes, correlated with antioxidant capacity related indicators. This study provides important information for broccoli flavonol genotypic variations and correlation with antioxidant capacity, and will facilitate the development of flavonol enriched cultivars in broccoli.

Keywords: Broccoli; Flavonols; Kaempferol; Quercetin; Antioxidant capacity; Flavonol synthase gene; Selenium

1. Introduction

Flavonoids are a class of naturally occurring secondary metabolites, which are synthesized in plants through the phenylpropanoid pathway. Flavonoids are widely distributed in plants and fulfil many important functions for plant growth and development (Cecilia, Alessio, Federico, Antonella, & Massimiliano, 2018; Ferreyra, Rius, & Casati, 2012). Based on their chemical structures, flavonoids are categorized into various subclasses. Flavonols belong to a subgroup of flavonoids with 3-hydroxyflavone backbone, and represent the most ubiquitous and abundant flavonoids in plant foods. In general, kaempferol and quercetin are the main representatives and have been widely studied due to their health-promoting functions. Both kaempferol and quercetin are known to have unique biological properties of anti-carcinogenic, antimicrobial, antidiabetic, and anti-inflammatory activities (Chen & Chen, 2013; Li et al., 2016). Epidemiological studies suggest that intake of foods enriched with kaempferol and quercetin can reduce the risk of some chronic diseases, such as cardiovascular diseases and cancers (Chen & Chen, 2013; Imran et al., 2019; Li et al., 2016).

Flavonoids, especially the flavonols, are important antioxidants that are associated with their abilities to suppress free radical formation, scavenge free radicals, and upregulate or protect antioxidant systems (Kumar & Pandey, 2013). They inhibit the enzymes associated with free radical production, reduce lipid peroxidation, and chelate metal ions in reducing free radical generation (Leopoldini, Russo, Chiodo, & Toscano, 2006). In plants, flavonoids contribute significantly to the antioxidant activity (Gu, Howell, Dunshea, & Suleria, 2019). Flavonols like quercetin was found to be associated with high antioxidant activity in vegetables (Frond et al., 2019). Kaempferol can also activate antioxidant enzymes (Doronicheva, Yasui, &

78 Sakurai, 2007). Considering the structure-activity relationship, flavonols that contains a galloyl
79 moiety usually have higher antioxidant activity than those without, and a B-ring
80 3′,4′,5′-trihydroxyl group can further improve their efficiency (Wolfe & Liu, 2008).

81 Broccoli (*Brassica oleracea* L. *italica*) is a cruciferous vegetable that contains copious
82 quantities of many nutrients and phytonutrients, including flavonols (Vasanthi, Mukherjee, &
83 Das, 2009). Its consumption has been steadily increasing year over year, in part due to its
84 containing of many health-promoting compounds (Herr & Buchler, 2010; Kaczmarek et al.,
85 2019). Investigation of genotypic variation is known to be an effective strategy for efficient
86 breeding crops with target traits. Large genetic variations of some health beneficial compounds
87 such as glucosinolates and *Se*-methylselenocysteine were reported in broccoli germplasm
88 (Kushad et al., 1999; Ramos, Yuan, Faquin, Guilherme, & Li, 2011). Broccoli is one of the
89 main flavonol sources in our diet. Kaempferol and quercetin in the glycoside forms represent
90 the main flavonols in broccoli (Vallejo, Tomás-Barberán, & Ferreres, 2004). Their stability
91 under different growth conditions and during marketing has been studied
92 (Gliszczyńska-Świgło, Kałużewicz, Lemańska, Knaflewski, & Tyrakowska, 2007; Molmann,
93 et al., 2015). However, the ability of different genotypes of broccoli in accumulating flavonols
94 and the correlation with total flavonoids and antioxidant capacity have not been fully
95 investigated.

96 In this study, we evaluated the variations of the two major flavonols kaempferol and
97 quercetin in leaves and florets of 15 broccoli accessions. We also examined the effects of
98 selenium (Se) treatment on these flavonol accumulations to see whether it is possible to
99 simultaneously accumulate multiple health-promoting compounds, because *Se*-biofortified

broccoli is known to synthesize functional forms of Se with enhanced chemopreventive activities. In addition, the total flavonoids, antioxidant capacity, and antioxidant enzyme activity were also investigated. The principal component analysis (PCA) and Pearson correlation were employed to reveal the correlations of flavonol levels with these associated indicators. The expression of genes involved in kaempferol and quercetin biosynthesis was investigated for a better understanding of the key genes controlling flavonols biosynthesis in broccoli.

2. Materials and methods

2.1. Plant materials

Fifteen accessions and varieties of broccoli (*Brassica oleracea* L. *italica*) were obtained from Plant Genetic Resources Unit at Geneva, NY, USA or as a gift (Superbroccoli) (Supplementary Table S1). For the production of leaf samples, seeds were surface-disinfected with 0.25% sodium hypochlorite and germinated by putting in the moistened roll sheets for 7 days at 25°C with a photoperiod of 16-h light and 8-h dark in an incubator. The young seedlings were then transferred to a container with Hoagland solution for conditioning in a greenhouse (Tian, Hui, Thannhauser, Pan, & Li, 2017). One week later, uniform seedlings were transferred to 2.2 L black pots containing the Hoagland solution and grown in the same greenhouse at 25°C with a 14-h light and 10-h dark photoperiod under constant aeration. The nutrient solution was changed every four days. After 12 days of culture, 60 plants (15 varieties

x 4 biological replicates) were harvested individually, and each replicate was weighed. The top three young leaves were collected. All the collected samples were immediately frozen in liquid nitrogen and stored at -80 ˚C for various analyses.

For the generation of floret samples, 60 plants (15 varieties x 4 biological replicates) were germinated in pots filled with soil mix and grown in a greenhouse. Florets were harvested individually when heads were fully formed and at market harvest maturity. The top center head samples were collected, ground into fine powder with mortars and pestles in liquid nitrogen, and stored at -80 ˚C for subsequent analyses.

In addition, three varieties that included Packman in the accession panel and Marathon and Diploma were grown in pots each filled with 6 dm^3 of soil mix for Se treatment at floret stage as described (Avila et al., 2013). When floral primordia began to form, 100 mL of 1.5 mM Na_2SeO_4 solution (equivalent to 25 μM) was applied to each pot. The application was performed twice a week for three weeks. When heads were fully formed and at market harvest maturity, the top center head samples were then harvested, ground into fine powder with mortars and pestles in liquid nitrogen, and stored at -80 ˚C.

2.2. Analysis of flavonols by Ultra-Performance Liquid Chromatography (UPLC)

Flavonols were extracted and analyzed following the methods as described (Rybarczyk-Plonska, Wold, Bengtsson, Borge, Hansen, & Hagen, 2016; Watkins, Hechler, & Muday, 2014) with some modifications. To extract flavonols, 150 mg of the powdered tissues were weighed out into a microcentrifuge tube and extracted with 450 μL of 80% methanol by

144 vortexing for 10 min and sonicating for 5 min. Following centrifugation at 14,000 g for 10 min,
145 450 µL of 2 M HCl were added to the supernatant and incubated for 45 min in a water bath at
146 70 ^{o}C to hydrolyze the glycosylated flavonoids and form aglycone flavonols. After hydrolysis,
147 equal volume of ethyl acetate was added, vortexed for 5 min, and centrifuged at 14,000 g for
148 10 min. The upper organic layer was collected, speed-vacuum dried, and resuspended in
149 acetone.

150 The extracted flavonol samples were analyzed using an ACQUITY UPLC equipped
151 with a BEH C18 column (2.1×50 mm, 1.7 µm) (Waters, Milford, MA, USA). Samples (2 µL)
152 were injected into the column and eluted using the mobile phases consisting of solvent A
153 (0.1% aqueous formic acid) and solvent B (0.1% formic acid in acetonitrile) in a linear gradient
154 of 15-85% solvent B over 4 min at flow rate of 0.6 mL min^{-1}. The column effluent was
155 monitored at 360 nm. Pure quercetin and kaempferol were obtained commercially (Sigma
156 Aldrich® Chemical Co., St. Louis, MO, USA) and used as standards for quantification.

157

158 *2.3. DPBA staining of kaempferol*

159

160 Kaempferol accumulation in roots was observed via diphenylboric acid 2-aminoethyl
161 ester (DPBA) staining according to the method as described (Vu et al., 2015). Briefly, 5-d old
162 seedlings were stained in a solution containing saturated DPBA (0.25%, w/v) and 0.02% (v/v)
163 Triton X-100 for 45 min, followed by three times of rinsing with distilled water. The
164 fluorescence of kaempferol in the samples was visualized under a Leica TCS SP5 laser
165 scanning confocal microscope (Leica Microsystems, Buffalo Grove, IL, USA).

166

167 *2.4. RNA extraction, reverse transcription and quantitative PCR analysis*

168

169 Total RNA was isolated from 100 mg of leaves using TRIzol reagent and
170 reverse-transcribed into cDNA using PrimeScript cDNA Synthesis Kit according to the
171 instruction of manufacturer (Takara Bio USA, Inc. Mountain View, CA, USA). Quantitative
172 real-time polymerase chain reaction (qRT-PCR) was performed with SYBR Green Master Mix
173 (Bio-Rad, Hercules, CA, USA) on CFX384 Touch Realtime PCR Detection System (Bio-Rad)
174 using gene-specific primers (Supplementary Table S2) as described previously (Cao et al.,
175 2019). The gene expression analysis was performed with three technical trials for each of the
176 three biological replicates.

177

178 *2.5. Cloning of flavonol synthase gene (FLS)*

179

180 The flavonol synthase gene was cloned from leaves of two high- and two low-
181 kaempferol varieties. Full-length *BoFLS* cDNAs were amplified using primers listed in
182 Supplementary Table S2 and cloned into pENTR vector (ThermoFish Scientific, Waltham,
183 MA, USA) for sequencing. Their sequences were aligned using Molecular Evolutionary
184 Genetics Analysis (MEGA) (Tamura, Dudley, Nei, & Kumar, 2007).

185

186 *2.6. Measurement of total flavonoid, antioxidant capacity, and total anthocyanin levels*

187

188 Total flavonoids and antioxidant capacity were analyzed according to the methods
189 described previously (Abu Bakar, Mohamed, Rahmat, & Fry, 2009) with some modifications.
190 To prepare the extracts, powdered broccoli tissues (100 mg) were mixed with 1 mL of 80%
191 methanol, vortexed for 10 min, and centrifuged at 12,000 g for 15 min. The supernatants were
192 collected and the pellets were extracted again. The combined supernatants were used for the
193 measurements.

194 To analyze total flavonoids, 50 μL of extracts were mixed with 225 μL of ddH_2O and
195 15 μL of 5% $NaNO_2$. The mixture was incubated at room temperature for 6 min, added with 30
196 μL of 10% $AlCl_3$, and incubated at room temperature for another 5 min, followed by addition
197 of 100 μL 1 M NaOH. The absorbance at wavelength of 510 nm was read. The total flavonoid
198 content was calculated against rutin standard with a gradient concentration series (0, 0.25, 0.50,
199 0.75, 1.0, 1.5 and 2.0 mg mL^{-1}), and expressed as mg rutin equivalents per 1 g sample (mg RE
200 g^{-1}).

201 The antioxidant capacity was determined by both 2,2-diphenyl-1-picrylhydrazyl (DPPH)
202 and ferric reducing/antioxidant power (FRAP) methods. The DPPH free radical scavenging
203 assay was performed according to the methods described (Abu Bakar et al, 2009). Aliquot (60
204 μL) of extracts or control (80% methanol) were mixed with 600 μL of 500 μM DPPH* in
205 ethanol. The mixture was vortexed vigorously, incubated in the dark for 30 min, and measured
206 spectrophotometrically at 517 nm. The free radical scavenging activity was calculated
207 according to the equation: Scavenging effect (%) = $[1-(A_{sample}/A_{control})] \times 100$. A gradient series
208 of ascorbic acid at concentrations from 0.05 to 0.25 mg mL^{-1} was also measured to generate a

209 standard curve and used to calculate antioxidant capacity. The data was expressed as mg
210 ascorbic acid equivalent antioxidant capacity per 1 g of sample (mg AEAC g^{-1}).

211 The FRAP assay was carried out using the method as described (Benzie & Strain, 1996)
212 with slight modifications. The FRAP reagent, prepared prior to use, contained a mixture of 300
213 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-*s*-triazine (TPTZ), and 20 mM $FeCl_3$ in a
214 10:1:1 ratio. One milliliter of 37 ˚C preheated FRAP reagent was mixed with 30 μL of plant
215 extracts and 90 μL of water, and incubated at 37 ˚C for 90 min. The absorbance at 593 nm was
216 read using a spectrophotometer. The absorbance difference between sample and blank control
217 was used to calculate the FRAP value against the standard curve, which was constructed with a
218 series of Fe^{2+} concentrations ranging from 100 to 1000 μM. The data was expressed as ferric
219 reducing ability per 1 gram of sample (μM Fe^{2+} g^{-1}).

220 Total anthocyanin content was extracted and measured following a previously reported
221 method (Tian, Xu, Liu, Xie, & Pan, 2016). Powdered broccoli leaf and floret samples (0.5 g
222 each) were added with 5 mL of acidified ethanol (85:15 of 95% ethanol: 1.5 M HCl) and
223 vortexed for 10 min. After centrifugation at 12,000 g for 10 min, the supernatants were
224 collected and measured spectrophotometrically at 535 nm. Anthocyanin content was calculated
225 using the equation: Amount (μg g^{-1} FW) = $(A_{535} \times V)/(98.2 \times W) \times 1000$. V and W represent
226 extract volume and sample weight, respectively. The value 98.2 represents the molar
227 absorptivity of cyaniding 3-galactoside at 535 nm.

228

229 *2.7. Antioxidant enzyme activity assays*

230

231 Plant extracts were prepared using the method described previously (Ramos et al, 2011).
232 Briefly, powdered broccoli sample (100 mg) was mixed with 1 mL of ice-cold extraction
233 buffer containing 50 mM phosphate-buffered saline (PBS) (pH 7.8), 0.2 mM
234 ethylenediaminetetraacetic acid (EDTA), and 0.3% (w/v) Triton X-100. The mixture was
235 vortexed well, kept on ice for 10 min, and centrifuged at 12,000 g for 10 min at 4°C. The
236 supernatant was quantified for protein levels by the Bradford method.

237 The antioxidant enzyme activity assays were performed essentially as described
238 previously (Ramos et al, 2011; Tian et al, 2017). To detect ascorbate peroxidase (APX) activity,
239 the reaction mixture (1 mL) contained 50 mM PBS (pH 7.0), 0.25 mM of reduced ascorbate,
240 12 mM H_2O_2 and 100 μL of plant extract. The reaction was started by addition of H_2O_2. The
241 reaction mixture with 100 μL of PBS solution to replace plant extract was used as the blank
242 control. The absorbance at 290 nm was recorded at an interval of 30 s for 2 min. The decrease
243 of A_{290} was used to calculate the APX activity.

244 To detect catalase (CAT) activity, the reaction reagent (1 mL) contained 50 mM PBS
245 (pH 7.6), 0.1 mM EDTA, 100 mM H_2O_2, and 100 μL of plant extract. The reaction was
246 initiated by addition of H_2O_2. The reaction mixture with 100 μL of PBS solution to replace
247 plant extract was used as the blank control. The absorbance at 240 nm was recorded at an
248 interval of 30 s for 2 min. The decrease of A_{240} was calculated and used to calculate the CAT
249 activity.

250 To detect superoxide dismutase (SOD) activity, the reaction mixture (1 mL) contained
251 50 mM PBS (pH 7.8), 0.1 mM EDTA, 13 mM methionine, 75 μM nitroblue tetrazolium (NBT),
252 2 μM riboflavin, and 100 μL of plant extract. Following addition of riboflavin, the reaction

mixture was illuminated for 15 min at the light intensity of 5000 lux. Two additional reaction mixtures with 100 μL of PBS solution instead of plant extract were used as controls, with one protected from light as blank control and the other illuminated for 15 min at the light intensity of 5000 lux. A_{560} was read and used to calculate the SOD activity following subtraction of the absorbance of illuminated control from sample. All experiments were performed with three biological replicates.

2.8. Statistical analysis

The data were presented as the means ± SD of three biological replicates for each sample. The significance difference among treatments was determined using IBM SPSS Statistics 20.0 with Duncan's multiple-range test (p<0.05). The heatmap was performed to evaluate the relationship among the physiological and biochemical parameters with the Pearson correlation coefficients (p<0.05). The "corrplot" package was accessed to generate the heatmap using the functions "corr" and "cor.mtest" to create coefficient and p-values matrices, respectively. Analysis of variance (ANOVA) was conducted to present the statistically significant correlations by inserting asterisks into heatmap cells.

3. Results

3.1 Plant growth varied greatly among the broccoli accessions

The broccoli accessions used consisted of both landraces and improved entries (Stansell et al., 2018). To compare growth characteristics, these accessions were grown hydroponically in the nutrient solution and their growth was evaluated by measuring plant biomass. The fresh biomass of 4-weeks old young plants ranged from 1.3 to 7.1 g per plant with over 5-fold difference between the highest and lowest accession (Supplementary Fig. S1). This data shows that the experimental broccoli materials represented a diverse collection of accessions with different growth.

3.2. Broccoli florets existed large variations in kaempferol and quercetin levels

Broccoli is known to accumulate kaempferol and quercetin in their glycoside forms (Vallejo et al, 2004). Their levels in leaves and florets of the broccoli accessions were evaluated after acid hydrolysis of glycosides. Large variations in kaempferol and quercetin content were observed among these accessions. The range of kaempferol accumulation varied from 108.0 to 274.3 μg g^{-1} FW, showing a 2.5-fold difference in leaves (Fig. 1A). The kaempferol levels were from 0.8 to 87.7 μg g^{-1} FW in florets, showing a much large variation (109-folds) in the edible tissue among these accessions (Fig. 1B). Broccoli accumulated higher levels of kaempferol in leaves than florets. The quercetin accumulation ranged from 8.0 to 32.2 μg g^{-1} FW in leaves with 4-fold difference, and from 1.8 to 29.0 μg g^{-1} FW in florets with 16-fold variations among these accessions (Fig. 1A and 1B). Overall, broccoli plants accumulated much more kaempferol than quercetin in leaves of all 15 accessions, though this was not the case in florets for some accessions. The ratios of kaempferol to quercetin amounts

297 varied from 4.4 to 27.9 in leaves and 0.4 to 4.4 in florets, indicating no correlations of
298 accumulation of these two flavonoid compounds in these broccoli accessions.

299 DPBA staining of root tissue is often used to visually detect kaempferol in plants (Vu et
300 al., 2015). The DPBA staining was also utilized in roots of two accessions with highest and
301 lowest kaempferol levels in leaves (i.e. accession 8 and 12, respectively). A much stronger
302 kaempferol-specific fluorescence intensity was detected in roots of accession 8 that with more
303 kaempferol than accession 12 with less kaempferol (Fig. 1C). The result indicates a rough
304 correlation between kaempferol levels in different tissues of a plant. Taken together, these
305 findings show different capacity of these broccoli accessions in accumulation of these
306 heath-beneficial flavonol compounds and suggest DPBA staining of roots as a rough screen for
307 high kaempferol accumulating varieties.

308

309 *3.3. Flavonol accumulation was directly associated with flavonol biosynthetic gene expression*

310

311 Flavonol biosynthetic pathway is well established (Fig. 2A). To gain a better
312 understanding of flavonol accumulation in broccoli, the transcript levels of several major genes
313 in the biosynthetic pathway and a regulator (i.e. *CHS, DFR, F3H, F3'H, FLS*, and *MYB*2) were
314 examined. Four accessions with two high (2 and 8) and two low (12 and 14) levels of
315 kaempferol in leaves were utilized for gene expression analysis. As shown in Figure 2B,
316 significant differences in expression of *F3H*, *F3'H*, and *FLS* were observed between both
317 accessions that accumulated high and two accessions that contained low levels of kaempferol.
318 *FLS* showed a largest 6-fold difference. Since *FLS* is responsible for kaempferol and other

flavonol synthesis (Fig. 2A), *FLS* CDS from these four accessions was cloned and its sequence difference among these four accessions was examined. A total of 24 SNPs in *FLS* CDS were observed among these four accessions (Supplementary Fig. 2A), suggesting abundant genetic variation in *FLS* gene at DNA level. Among the 24 SNPs, only one caused an amino acid change with Val or Ala between different accessions (Supplementary Fig. 2B). However, this variation does not correlate with kaempferol accumulation. The results suggest that the difference in kaempferol accumulation was likely caused at transcription level of *FLS*.

3.4. Kaempferol and quercetin levels were not influenced by selenium treatment

Broccoli as a selenium (Se) secondary accumulator synthesizes and accumulates anticarcinogenic compounds such as *Se*-methylselenocysteine when grown in media containing Se (Avila et al., 2013; Lyi, Heller, Rutzke, Welch, Kochian, & Li, 2005; Ramos et al, 2011). To examine whether Se treatment affected flavonol accumulation, kaempferol and quercetin levels were quantified in florets and leaves of three broccoli cultivars treated with or without 25 μM Na_2SeO_4, a concentration and form of Se that promote *Se*-methylselenocysteine production and cause no adversative impact on plant growth and development (Avila et al., 2013; Lyi et al, 2005; Tian et al., 2018). As shown in Figure 3A, Se treatment did not reduce kaempferol levels in both leaf and floret tissues of these broccoli cultivars, indicating a potential to enhance bioactive Se compounds without impact on kaempferol level. Consistently, leaves contained more kaempferol than florets (Fig. 3A).

340 The level of quercetin was also examined in these cultivars. No significant difference in
341 quercetin levels were observed among these cultivars (Fig. 3B). When treated with Se, the
342 cultivar Marathon showed a significantly reduced content of quercetin in leaves (p<0.05),
343 while the other two cultivars did not give statistically significant change. In contrast, Se
344 treatment showed no reduced quercetin levels and with a significant increase in florets of one
345 variety (Fig. 3B). Slightly lower levels of quercetin were detected in florets than leaves for
346 these cultivars (Fig. 3B). Taken together, these results show that kaempferol and quercetin
347 levels in general were not greatly affected by Se application, indicating the possibility of
348 simultaneous enrichment of health-beneficial flavonol and Se compounds.
349
350 *3.5. Total flavonoids, anthocyanins and antioxidant capacity showed different levels of*
351 *genotypic variations*
352
353 In addition to flavonols kaempferol and quercetin, the total flavonoid levels were also
354 examined in leaves and florets of the 15 broccoli accessions. The total flavonoid content varied
355 across the two tissues and different accessions, ranging from 115.7 to 310.9 μg RE g^{-1} FW in
356 leaves and 31.1 to 103.9 μg RE g^{-1} FW in florets (Fig. 4A). A difference of 2.5-folds and
357 3.3-folds was detected in leaves and florets, respectively, among accessions with low and high
358 levels of total flavonoids. Interestingly, the variations in total flavonoid levels showed no
359 apparent correlations with the variation in kaempferol and quercetin content among these
360 accessions (Fig. 1 and Fig. 4A), indicating that the high level of total flavonoids does not
361 necessarily reflect high kaempferol or quercetin content in a particular variety.

362 Anthocyanins are a subgroup of flavonoid compounds and give plant tissues color.
363 Examination of total anthocyanin content revealed that all accessions contained similar levels
364 in leaves, while a 3.5-fold difference was observed in florets between accessions with the
365 highest and lowest content (Fig. 4B). This result shows that anthocyanin content did not vary
366 greatly in green leaves but in florets among different broccoli accessions. These findings
367 suggest that florets are responsive to the environmental and growth influences for anthocyanin
368 production in broccoli.

369 The total antioxidant capacity in leaves and florets of broccoli accessions was
370 determined by both DPPH and FRAP methods. The DPPH values showed over 2-fold changes,
371 ranging from 1.6 to 3.5 mg AEAC g^{-1} FW in leaves of broccoli accessions, while much high
372 DPPH values were observed in a few accessions than others in florets (Fig. 4C). The FRAP
373 values also showed over 2-fold changes in leaves, but very similar in florets among these
374 broccoli accessions (Fig. 4D). A general coincidence in total antioxidant capacity measured by
375 the two DPPH and FRAP methods was observed in leaves, but not in florets of some
376 accessions.

377

378 *3.6. Antioxidant enzyme activities varied in broccoli accessions*

379

380 The activities of antioxidant enzymes, i.e., APX, CAT, and SOD, in these broccoli
381 accessions were examined. Overall, the activities of these enzymes varied depending on
382 accessions. The APX value was 6.2 and 19.4 μmol min^{-1} mg^{-1} proteins in leaves of accessions
383 1 and 2, respectively, showing a 3-fold difference between the lowest and highest accessions,

384 while it varied from 1.7 to 11.3 μmol min^{-1} mg^{-1} proteins in florets with a 6-fold difference
385 (Fig. 5A). APX activity was generally lower in florets, though there were some accessions with
386 similar activity in leaves and florets (Fig. 5A). For CAT, approximately 2-fold activity
387 difference was observed in leaves, but not much variation was found in florets among these
388 broccoli accessions (Fig. 5B). There was about 2-fold difference for SOD activity in leaves,
389 and a 5-fold difference in florets with the lowest and highest accessions (Fig. 5C).
390 Interestingly, APX and CAT activities were generally higher in leaves, while SOD activity was
391 lower in leaves than florets. These results indicate the complexity in antioxidant enzymes
392 between leaves and florets of a specific accession or among accessions.
393
394 *3.7 Flavonol accumulation was correlated with antioxidant-related attributes*
395
396 The correlations between flavonols and various parameters analyzed in florets and leaves
397 of these accessions were examined and shown in Figure 6. In florets, positive correlation was
398 observed between anthocyanins and FRAP, and between total flavonoids, DPPH, APX, and
399 SOD (p<0.05), while CAT was negatively correlated with quercetin and anthocyanins (p<0.05)
400 (Fig. 6A). For broccoli leaves, anthocyanins were positively correlated with quercetin, FRAP
401 and SOD, and total flavonoids were correlated with kaempferol, DPPH and FRAP (p<0.05).
402 Moreover, the expression of genes controlling flavonol biosynthesis presented a very strong
403 correlation with kaempferol, flavonoid and antioxidant capacities (p<0.05) (Fig. 6B).
404 Comparing kaempferol and quercetin, there was a weak negative correlation in florets and a
405 weak positive correlation in leaves, but both failed to pass the statistical significance level

406 (p>0.05) (Fig. 6A and B), indicating that accumulation of these two flavonols does not interact
407 with each other in either florets or leaves. Between two tissues of leaves and florets, a positive
408 correlation was observed in kaempferol to give a correlation coefficient of 0.586 (p<0.05), but
409 not in quercetin and total flavonoids (with a correlation coefficient of -0.100 and 0.101,
410 respectively) (p>0.05). These results suggest that flavonols, as well as key biosynthesis
411 pathway genes, exist correlations with antioxidant capacity-related attributes at different
412 extents in broccoli tissues.

413 Principal component analysis (PCA) is a broadly used multivariate technique that
414 extracts important information from a data table with observations of several inter-correlated
415 variables and displays the pattern of similarity of different variables (Abdi & Williams, 2010).
416 PCA revealed that PC1 and PC2 explained 25.9 and 23.1% of total variations (Fig. 6C and D),
417 and these components were mainly the flavonols and antioxidant capacity related indicators
418 (Supplementary Table S3). When the expressions of flavonol biosynthesis genes were included
419 for PCA, the PC1 accounted for 62.57% of total variations between high and low kaempferol
420 accessions (Supplementary Fig. 3, Supplementary Table S4).

421

422 **4. Discussion**

423

424 The flavonols kaempferol and quercetin have gained increasing interests due to their
425 health-promoting properties (Chen & Chen, 2013; Li et al., 2016). Broccoli is known to mainly
426 accumulate these two flavonols (Gliszczyńska-Świgło et al, 2007; Molmann et al., 2015;
427 Vallejo et al, 2004). Thus, this attribute along with its multiple health-benefit nutrients and

phytonutrients makes broccoli an excellent functional food. In this study, 15 broccoli accessions consisted of both landraces and improved entries (Stansell et al., 2018) were examined for their flavonol levels and antioxidant associated attributes.

In broccoli, flavonol content was reported to be affected by floret developmental stage (Krumbein, Saeger-Fink, & Schonhof, 2007), temperature (Molmann et al., 2015), and radiation treatments (Gliszczyńska-Świgło et al, 2007; Molmann et al., 2015; Rybarczyk-Plonska et al, 2016). Here, we showed that great genotypic variations in flavonol content existed in florets among these broccoli accessions (Fig. 1). The kaempferol levels varied over 100-folds, and the quercetin amounts differed over 16-folds. The kaempferol-quercetin ratios ranged between 0.4 to 5.5 in broccoli florets, consistent with previous reports (Gliszczyńska-Świgło et al, 2007; Krumbein et al, 2007). In addition, we examined flavonols in leaves and found that broccoli leaves accumulated much higher levels of kaempferol, but similar quercetin, with less genotypic variation ($\leq$4-folds) than florets (Fig. 1). As a result, there were much higher kaempferol to quercetin ratios in leaves varying between 4.4 to 27.9 among different accessions. While kaempferol was the predominant flavonol in leaves, both kaempferol and quercetin accumulated at relatively comparable amounts in florets of various accessions. In addition, DPBA staining of roots showed a good correlation with kaempferol accumulation in other plant tissues, which may provide a good indicator for identifying high kaempferol accumulating varieties at early stages of breeding.

Flavonol biosynthesis shares the upstream biosynthetic pathway with other flavonoids and separates at dihydroflavonol (Fig. 2A). Thus, it shares some common key enzymes that include chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H),

and flavonoid 3′-hydroxylase (F3'H). FLS catalyzes the conversion of dihydroflavonols into flavonols. In *Brassica napus*, flavonol biosynthesis was found to be promoted by high sucrose, mannitol and NaCl (Vu et al., 2015), similarly as shown in other plants (Fini, Brunetti, Di Ferdinando, Ferrini, & Tattini, 2011). The flavonol accumulation in the *B. napus* is correlated with the *BnFLS* gene expression (Vu et al., 2015). This implies that the *FLS* gene plays a key role in flavonol biosynthesis in *Brassica* crops. Consistently, when examining the expression of flavonol biosynthetic genes, we observed significant difference in *FLS* gene expression along with *F3H* and *F3'H* between high and low kaempferol producing accessions. The much high level of *FLS* transcription in the high kaempferol accession shows its important role for flavonol accumulation in broccoli as in *B. napus* (Vu et al., 2015). Interestingly, while *FLS* gene expression correlated with kaempferol or total flavonol levels, it showed less correlation with the accumulation of quercetin, indicating that additional factors contribute to the regulation of other individual flavonol content in broccoli.

Many flavonoids are strong antioxidants (Kumar & Pandey, 2013; Terao, 2009). While the total antioxidant capacity as measured by DPPH and FRAP showed no great differences in both leaves and florets among these broccoli accessions, kaempferol and total flavonoids were generally consistent with the antioxidant capacity and activities of antioxidant enzymes as revealed by Pearson correlation and PCA (Fig. 6). It has been documented that antioxidant capacity is contributed by many biologically active biomolecules, such as vitamin C, phenolic compounds, and carotenoids in plants (Apak, Ozyurek, Guclu, & Capanoglu, 2016). The antioxidant capacity was to some extent correlated with flavonoids and kaempferol, implying

that total flavonoid and kaempferol contribute partly and the antioxidant capacity is a composite result of complex factors.

Se-biofortified broccoli is a promising approach to supply bioactive forms of Se with strong anticarcinogenic activity (Avila et al., 2013; Lyi et al, 2005; Ramos et al, 2011). We observed that Se-biofortification did not compromise the accumulation of major flavonols kaempferol and quercetin in the broccoli varieties tested (Fig. 3). Thus, it is possible to simultaneously produce bioactive Se compounds and maintain the flavonol levels in edible parts of broccoli to further enhance its chemopreventive properties.

In summary, we present a comprehensive evaluation of broccoli germplasm for genotypic variations with flavonols along with total flavonoids and antioxidant capacity. We found great genetic variation in those major flavonols kaempferol and quercetin among the broccoli accessions tested. The information would be useful in selecting and developing broccoli cultivars for high flavonol content with enhanced health-promoting properties.

CRediT authorship contribution statement

Yongbo Duan: Conceptualization, Investigation, Methodology, Validation, Writing – original draft. **Franklin Eduardo Melo Santiago:** Investigation, Methodology, Validation. **Andre Rodrigues dos Reis:** Data analysis. **Marislaine A. de Figueiredo:** Methodology. **Suping Zhou:** Methodology. **Theodore W. Thannhauser:** Methodology, Writing - review & editing. **Li Li:** Conceptualization, Validation, Supervision, Writing- review & editing.

Declaration of Competing Interests

493 The authors declare that they have no known competing financial interests or personal
494 relationships that could have appeared to influence the work reported in this paper.
495
496 **Acknowledgement**
497 The authors would like to thank Dr. Hui Yuan for assistance with fluorescence
498 observation of DPBA-stained broccoli roots, Ms. Tara Fish for assistance with UPLC analysis,
499 and Drs. Yao-Guo Qin and Hua-Qiang Tan for their assistance in collecting plant samples. DY
500 acknowledges the support of Anhui Provincial Educational Department, China (gwfx2017038).
501 This work was supported by the USDA-ARS fund.
502
503 **Supplementary data**
504 Supplementary data to this article can be found online
505
506

References

Abdi, H., & Williams, L. J. (2010). Principal component analysis. *WIREs Computational Statistics, 2*(4), 433-459. https://doi.org/10.1002/wics.101

Abu Bakar, M. F., Mohamed, M., Rahmat, A., & Fry, J. (2009). Phytochemicals and antioxidant activity of different parts of bambangan (*Mangifera pajang*) and tarap (*Artocarpus odoratissimus*). *Food Chemistry, 113*(2), 479-483. https://doi.org/10.1016/j.foodchem.2008.07.081

Apak, R., Ozyurek, M., Guclu, K., & Capanoglu, E. (2016). Antioxidant activity/capacity measurement. 1. Classification, physicochemical principles, mechanisms, and electron transfer (ET)-based assays. *Journal of Agricultural and Food Chemistry, 64*(5), 997-1027. https://doi.org/10.1021/acs.jafc.5b04739

Avila, F. W., Faquin, V., Yang, Y., Ramos, S. J., Guilherme, L. R., Thannhauser, T. W., & Li, L. (2013). Assessment of the anticancer compounds Se-methylselenocysteine and glucosinolates in Se-biofortified broccoli (*Brassica oleracea* L. var. *italica*) sprouts and florets. *Journal of Agricultural and Food Chemistry, 61*(26), 6216-6223. https://doi.org/10.1021/jf4016834

Benzie, I. F., & Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Analytical Biochemistry, 239*(1), 70-76. https://doi.org/10.1006/abio.1996.0292

Cao, H., Luo, H., Yuan, H., Eissa, M. A., Thannhauser, T. W., Welsch, R., Hao, Y. J., Cheng, L., & Li, L. (2019). A Neighboring Aromatic-Aromatic Amino Acid Combination Governs Activity Divergence between Tomato Phytoene Synthases. *Plant Physiol, 180*(4), 1988-2003. https://doi.org/10.1104/pp.19.00384

529 Cecilia, B., Alessio, F., Federico, S., Antonella, G., & Massimiliano, T. (2018). Modulation of
530 phytohormone signaling: A primary function of flavonoids in plant-environment interactions.
531 *Frontiers in Plant Science, 9,* 1042. https://doi.org/10.3389/fpls.2018.01042
532 Chen, A. Y., & Chen, Y. C. (2013). A review of the dietary flavonoid, kaempferol on human health
533 and cancer chemoprevention. *Food Chemistry, 138*(4), 2099-2107.
534 https://doi.org/10.1016/j.foodchem.2012.11.139
535 Doronicheva, N., Yasui, H., & Sakurai, H. (2007). Chemical structure-dependent differential effects
536 of flavonoids on the catalase activity as evaluated by a chemiluminescent method. *Biological &*
537 *Pharmaceutical Bulletin, 30*(2), 213-217. https://doi.org/10.1248/bpb.30.213
538 Ferreyra, M. a. L. F., Rius, S. P., & Casati, P. (2012). Flavonoids: biosynthesis, biological functions
539 and biotechnological applications. *Frontiers in Plant Science, 3*(222), 222.
540 https://doi.org/10.3389/fpls.2012.00222
541 Fini, A., Brunetti, C., Di Ferdinando, M., Ferrini, F., & Tattini, M. (2011). Stress-induced flavonoid
542 biosynthesis and the antioxidant machinery of plants. *Plant Signal & Behavior, 6*(5), 709-711.
543 https://doi.org/10.4161/psb.6.5.15069
544 Frond, A. D., Iuhas, C. I., Stirbu, I., Leopold, L., Socaci, S., Andreea, S., Ayvaz, H., Andreea, S.,
545 Mihai, S., & Diaconeasa, Z. A. (2019). Phytochemical characterization of five edible
546 purple-reddish vegetables: anthocyanins, flavonoids, and phenolic acid derivatives. *Molecules,*
547 *24*(8), 1536. https://doi.org/10.3390/molecules24081536
548 Gliszczyńska-Świgło, A., Kałużewicz, A., Lemańska, K., Knaflewski, M., & Tyrakowska, B. (2007).
549 The effect of solar radiation on the flavonol content in broccoli inflorescence. *Food Chemistry,*
550 *100*(1), 241-245. https://doi.org/10.1016/j.foodchem.2005.09.048s

551 Gu, C., Howell, K., Dunshea, F. R., & Suleria, H. A. R. (2019). LC-ESI-QTOF/MS characterisation
552 of phenolic acids and flavonoids in polyphenol-rich fruits and vegetables and their potential
553 antioxidant activities. *Antioxidants, 8*(9), 405. https://doi.org/10.3390/antiox8090405
554 Herr, I., & Buchler, M. W. (2010). Dietary constituents of broccoli and other cruciferous vegetables:
555 implications for prevention and therapy of cancer. *Cancer Treatment Reviews, 36*(5), 377-383.
556 https://doi.org/10.1016/j.ctrv.2010.01.002
557 Imran, M., Salehi, B., Sharifi-Rad, J., Aslam Gondal, T., Saeed, F., Imran, A., Shahbaz, M., Tsouh
558 Fokou, P. V., Umair Arshad, M., Khan, H., Guerreiro, S. G., Martins, N., & Estevinho, L. M.
559 (2019). Kaempferol: A key emphasis to its anticancer potential. *Molecules, 24*(12), 2277.
560 https://doi.org/10.3390/molecules24122277
561 Kaczmarek, J. L., Liu, X., Charron, C. S., Novotny, J. A., Jeffery, E. H., Seifried, H. E., Ross, S. A.,
562 Miller, M. J., Swanson, K. S., & Holscher, H. D. (2019). Broccoli consumption affects the human
563 gastrointestinal microbiota. *Journal of Nutritional Biochemistry, 63*, 27-34.
564 https://doi.org/10.1016/j.jnutbio.2018.09.015
565 Krumbein, I., Saeger-Fink, H., & Schonhof, I. (2007). Changes in quercetin and kaempferol
566 concentrations during broccoli head ontogeny. *Journal of Applied Botany and Food Quality, 81*,
567 136 - 139. https://doi.org/10.1086/521686
568 Kumar, S., & Pandey, A. K. (2013). Chemistry and biological activities of flavonoids: An overview.
569 *The Scientific World Journal, 2013*, 162750. https://doi.org/10.1155/2013/162750
570 Kushad, M. M., Brown, A. F., Kurilich, A. C., Juvik, J. A., Klein, B. P., Wallig, M. A., & Jeffery, E.
571 H. (1999). Variation of glucosinolates in vegetable crops of *Brassica oleracea*. *Journal of*
572 *Agricultural and Food Chemistry, 47*(4), 1541-1548. https://doi.org/10.1021/jf980985s

573 Leopoldini, M., Russo, N., Chiodo, S., & Toscano, M. (2006). Iron chelation by the powerful
574 antioxidant flavonoid quercetin. *Journal of Agricultural and Food Chemistry, 54*(17), 6343-6351.
575 https://doi.org/10.1021/jf060986h
576 Li, Y., Yao, J., Han, C., Yang, J., Chaudhry, M. T., Wang, S., Liu, H., & Yin, Y. (2016). Quercetin,
577 Inflammation and Immunity. *Nutrients, 8*(3), 167. https://doi.org/10.3390/nu8030167
578 Lyi, S. M., Heller, L. I., Rutzke, M., Welch, R. M., Kochian, L. V., & Li, L. (2005). Molecular and
579 biochemical characterization of the selenocysteine Se-methyltransferase gene and
580 Se-methylselenocysteine synthesis in broccoli. *Plant Physiology, 138*(1), 409-420.
581 https://doi.org/10.1104/pp.104.056549
582 Molmann, J. A., Steindal, A. L., Bengtsson, G. B., Seljasen, R., Lea, P., Skaret, J., & Johansen, T. J.
583 (2015). Effects of temperature and photoperiod on sensory quality and contents of glucosinolates,
584 flavonols and vitamin C in broccoli florets. *Food Chemistry, 172*, 47-55.
585 https://doi.org/10.1016/j.foodchem.2014.09.015
586 Ramos, S. J., Yuan, Y., Faquin, V., Guilherme, L. R., & Li, L. (2011). Evaluation of genotypic
587 variation of broccoli (*Brassica oleracea* var. *italic*) in response to selenium treatment. *Journal of*
588 *Agricultural and Food Chemistry, 59*(8), 3657-3665. https://doi.org/10.1021/jf104731f
589 Rybarczyk-Plonska, A., Wold, A. B., Bengtsson, G. B., Borge, G. I. A., Hansen, M. K., & Hagen, S.
590 F. (2016). Flavonols in broccoli (*Brassica oleracea* L. var. *italica*) flower buds as affected by
591 postharvest temperature and radiation treatments. *Postharvest Biology and Technology, 116*,
592 105-114. https://doi.org/10.1016/j.postharvbio.2015.12.023
593 Stansell, Z., Hyma, K., Fresnedo-Ramirez, J., Sun, Q., Mitchell, S., Bjorkman, T., & Hua, J. (2018).
594 Genotyping-by-sequencing of *Brassica oleracea* vegetables reveals unique phylogenetic patterns,

595 population structure and domestication footprints. *Horticulture Research, 5,* 38.
596 https://doi.org/10.1038/s41438-018-0040-3
597 Tamura, K., Dudley, J., Nei, M., & Kumar, S. (2007). MEGA4: Molecular Evolutionary
598 Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol. 24*(8),1596-1599.
599 doi:10.1093/molbev/msm092
600 Terao, J. (2009). Dietary flavonoids as antioxidants. *Forum of Nutrition, 61*(61), 87.
601 http://dx.doi.org/10.1159/000212741
602 Tian, M., Hui, M., Thannhauser, T. W., Pan, S., & Li, L. (2017). Selenium-induced toxicity is
603 counteracted by sulfur in broccoli (*Brassica oleracea* L. var. *italica*). *Frontiers Plant Science, 8*,
604 1425. https://doi.org/10.3389/fpls.2017.01425
605 Tian, M., Xu, X., Liu, Y., Xie, L., & Pan, S. (2016). Effect of Se treatment on glucosinolate
606 metabolism and health-promoting compounds in the broccoli sprouts of three cultivars. *Food*
607 *Chemistry, 190*, 374-380. https://doi.org/10.1016/j.foodchem.2015.05.098
608 Tian, M., Yang, Y., Avila, F. W., Fish, T., Yuan, H., Hui, M., Pan, S., Thannhauser, T. W., & Li, L.
609 (2018). Effects of selenium supplementation on glucosinolate biosynthesis in broccoli. *Journal of*
610 *Agricultural and Food Chemistry, 66*(30), 8036-8044. https://doi.org/10.1021/acs.jafc.8b03396s
611 Vallejo, F., Tom ás-Barber án, F. A., & Ferreres, F. (2004). Characterisation of flavonols in broccoli
612 (Brassica oleracea L. var. italica) by liquid chromatography-UV diode-array
613 detection-electrospray ionisation mass spectrometry. *Journal of Chromatography A, 1054*(1-2),
614 181-193. https://doi.org/10.1016/j.chroma.2004.05.045

615 Vasanthi, H. R., Mukherjee, S., & Das, D. K. (2009). Potential health benefits of broccoli- A
616 chemico-biological overview. *Mini Reviews in Medicinal Chemistry, 9*(6), 749-759.
617 http://doi.org/10.2174/138955709788452685
618 Vu, T. T., Jeong, C. Y., Nguyen, H. N., Lee, D., Lee, S. A., Kim, J. H., Hong, S. W., & Lee, H.
619 (2015). Characterization of *Brassica napus* flavonol synthase involved in flavonol biosynthesis in
620 *Brassica napus* L. *Journal of Agricultural and Food Chemistry, 63*(35), 7819-7829.
621 https://doi.org/10.1021/acs.jafc.5b02994
622 Watkins, J. M., Hechler, P. J., & Muday, G. K. (2014). Ethylene-induced flavonol accumulation in
623 guard cells suppresses reactive oxygen species and moderates stomatal aperture. *Plant*
624 *Physiology, 164*(4), 1707-1717. https://doi.org/10.1104/pp.113.233528
625 Winkler, S., Faragher, J., Franz, P., Imsic, M., & Jones, R. (2007). Glucoraphanin and flavonoid
626 levels remain stable during simulated transport and marketing of broccoli (*Brassica oleracea* var.
627 *italica*) heads. *Postharvest Biology and Technology, 43*(1), 89-94.
628 https://doi.org/10.1016/j.postharvbio.2006.08.001
629 Wolfe, K. L., & Liu, R. H. (2008). Structure-activity relationships of flavonoids in the cellular
630 antioxidant activity assay. *Journal of Agricultural and Food Chemistry, 56*(18), 8404-8411.
631 https://doi.org/10.1021/jf8013074
632 Zhou, X., McQuinn, R., Fei, Z., Wolters, A. A., J, V. A. N. E., Brown, C., Giovannoni, J. J., & Li, L.
633 (2011). Regulatory control of high levels of carotenoid accumulation in potato tubers. *Plant Cell*
634 *Environment, 34*(6), 1020-1030. https://doi.org/10.1111/j.1365-3040.2011.02301.x

635

636 **Figure legends**

637 **Fig. 1.** Kaempferol and quercetin levels in various broccoli accessions. (A) Kaempferol and
638 quercetin levels in leaves. (B) Kaempferol and quercetin levels in florets. (C) Representative
639 images of DPBA staining on roots of 5 days old broccoli plants from genotype 8 and 12. The
640 numbers indicate the germplasm ID numbers listed in Supplementary Table S1. Bars
641 represent 3 mm.

642

643 **Fig. 2.** Flavonol biosynthetic pathway and relative expression of the pathway genes. (A)
644 Outline of flavonol biosynthetic pathway. (B) qRT-PCR analysis of expression of flavonol
645 biosynthetic genes in two high (2 and 8) vs two low (12 and 14) kaempferol genotypes. Error
646 bars indicate standard error of the mean (n=3). The different lowercases stand for significant
647 difference between accessions for each gene (p<0.05). CHS, chalcone synthase; DFR,
648 dihydroflavonol 4-reductase; F3H, flavanone hydroxylase; F3'H, flavonoid 3'-hydroxylase;
649 FLS, flavonol synthase; Myb2, MYB transcription factor 2.

650

651 **Fig. 3.** Effect of selenium on kaempferol and quercetin levels. (A) Effect of selenium on
652 kaempferol levels in leaves and florets of three broccoli cultivars treated with or without 25
653 μM Na_2SeO_4. (B) Effect of selenium on quercetin levels. Error bars indicate standard error of
654 the mean (n=3). * Represents significant difference (p <0.05)

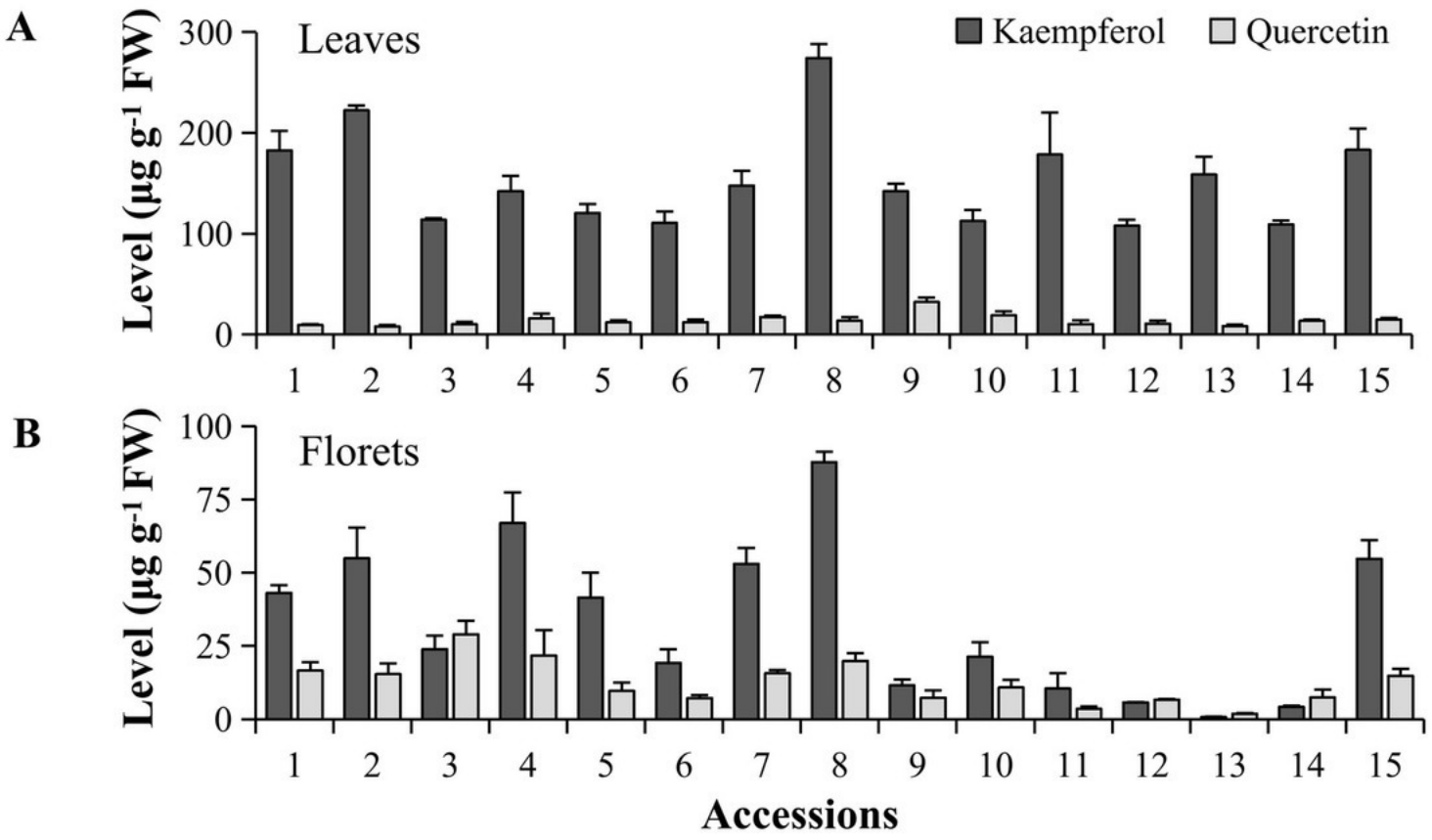
655

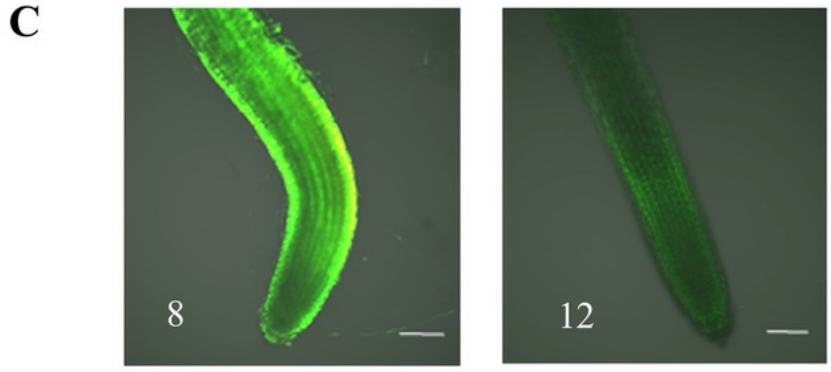
656 **Fig. 4.** Total levels of flavonoids, anthocyanins, and antioxidant capacities in leaves and florets of
657 different broccoli accessions. (A) Flavonoid levels. (B) Anthocyanin levels. (C) Antioxidant capacity

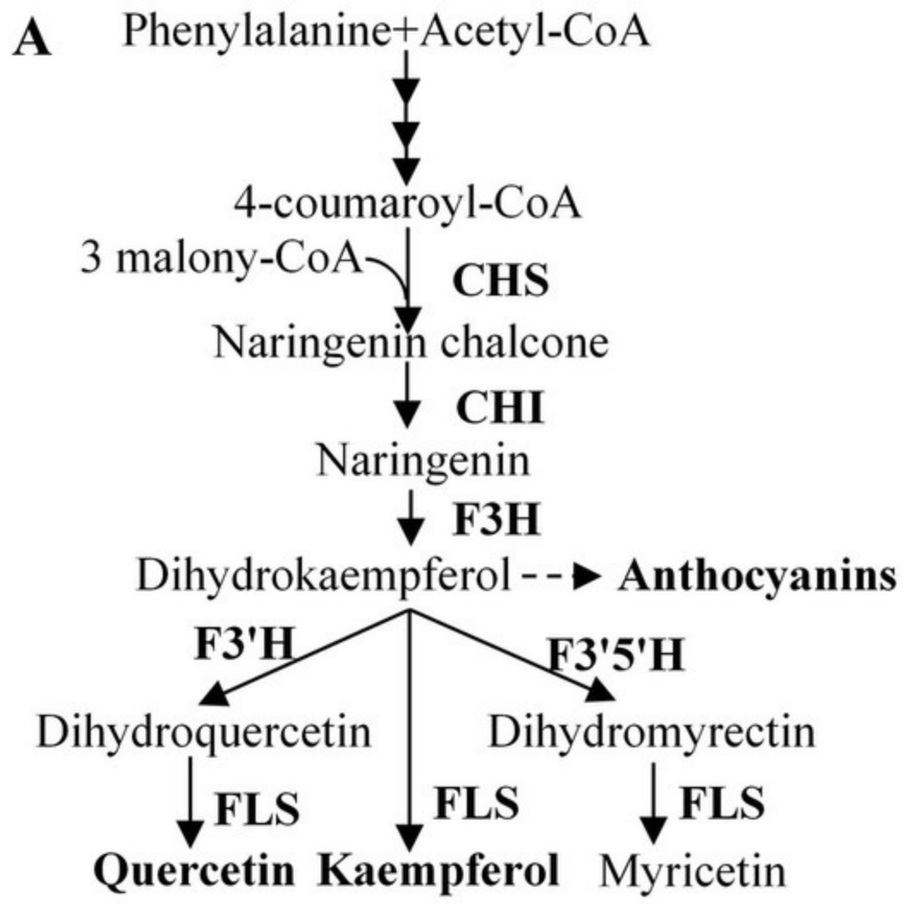
by DPPH assay. (D) Antioxidant capacity by FRAP assay. Error bars indicate standard error of the mean (n=3).

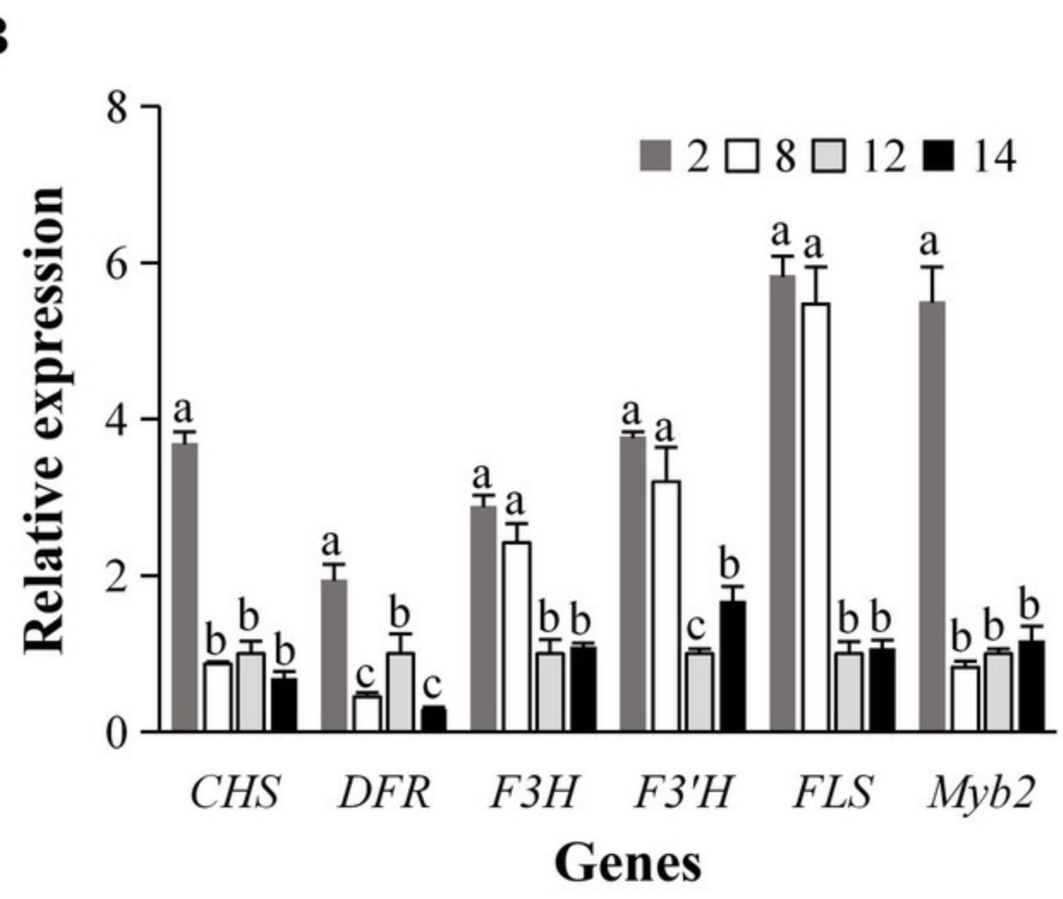
Fig. 5. Antioxidant enzyme activities in leaves and florets of different broccoli accessions. (A) Ascorbate peroxidase (APX) activity. (B) Catalase (CAT) activity. (C) Superoxide dismutase (SOD) activity. Error bars indicate standard error of the mean (n=3).

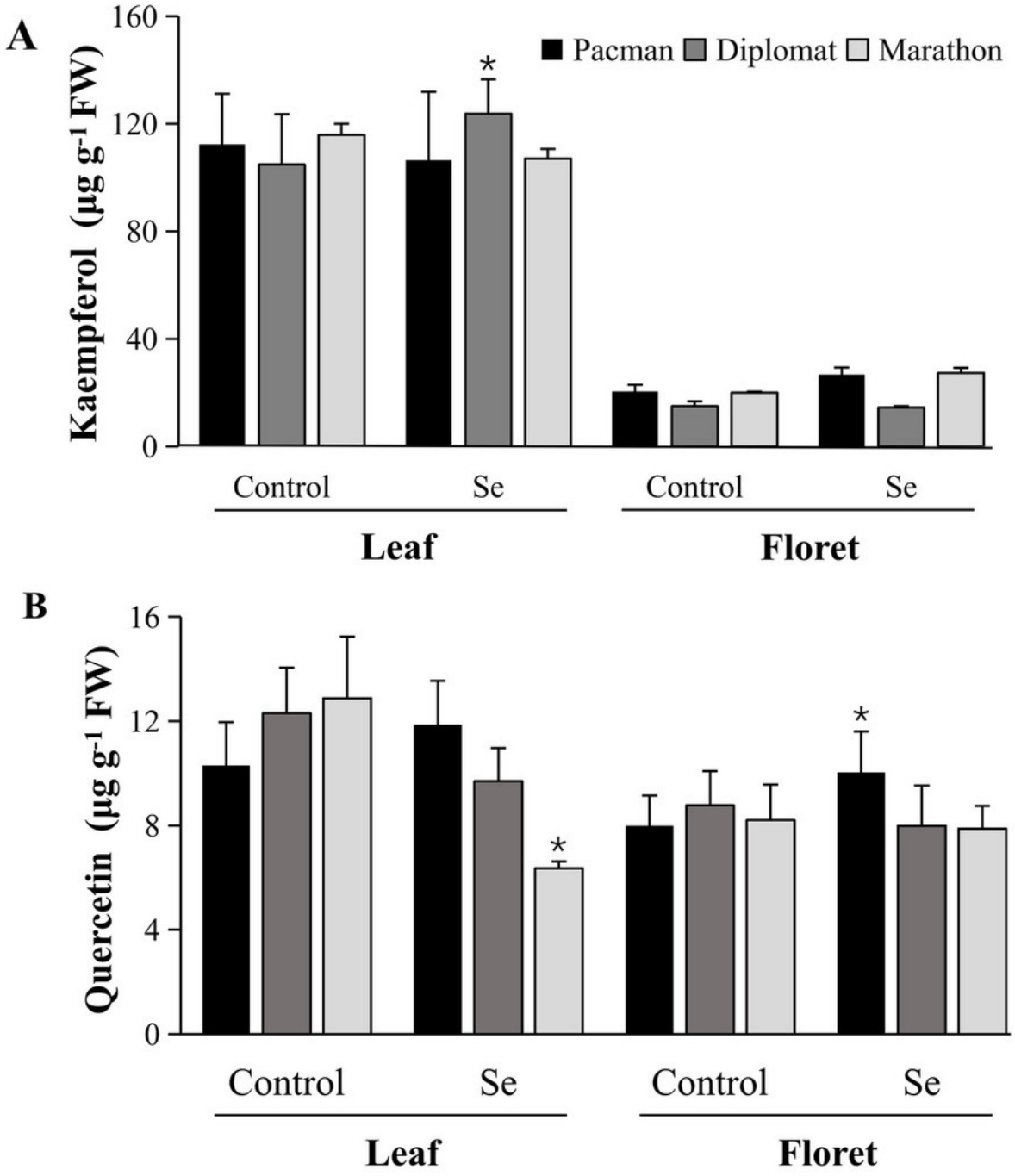
Fig. 6. Heatmap of Pearson correlation coefficients obtained from variables of broccoli plants. (A) Corresponds to physiological parameters analyzed in florets, and (B) analyzed in leaves. * indicates significant correlation (p <0.05). (C) PCA triplot showing the loading of each genotype score (points) and groups (circles). (D) PCA triplot showing the loading of plant attributes (arrows). Nearby points correspond to observations with similar scores in the PCA components. The length of the arrows indicates the variation of attributes in the component, while the points between them indicate their correlations. Kaemp: kaempferol, Quer: quercetin, Anth: anthocyanin, Fla: flavonoids, FRAP: ferric reducing antioxidant power, DPPH: 2,2-diphenyl-1-picryl-hydrazyl-hydrate, SOD: superoxide dismutase, CAT: catalase, APX: ascorbate peroxidase.

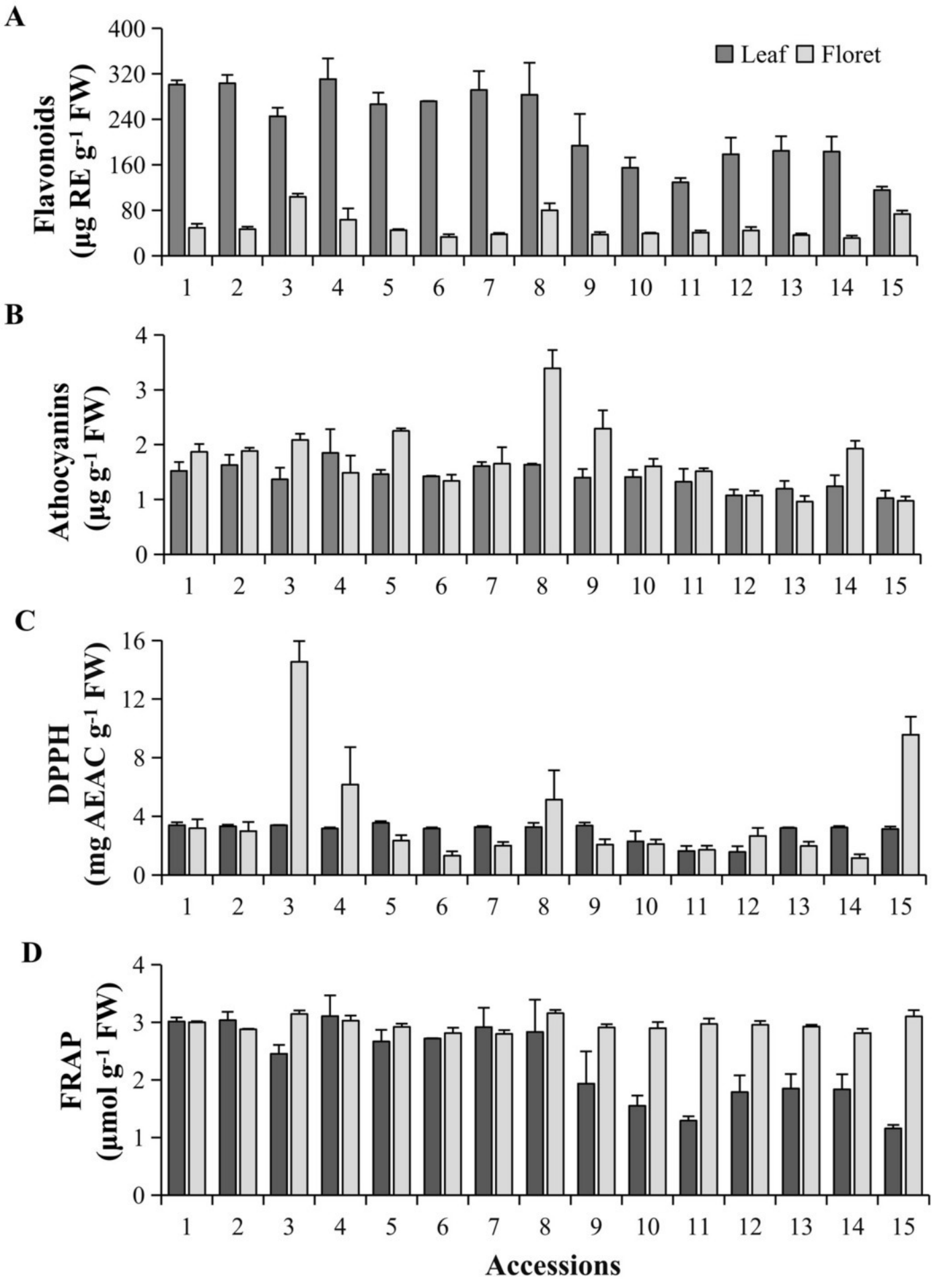

A
Leaves
Kaempferol
Quercetin
Level (µg g-1 FW)
300
200
100
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
B
Florets
Level (µg g-1 FW)
100
75
50
25
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Accessions

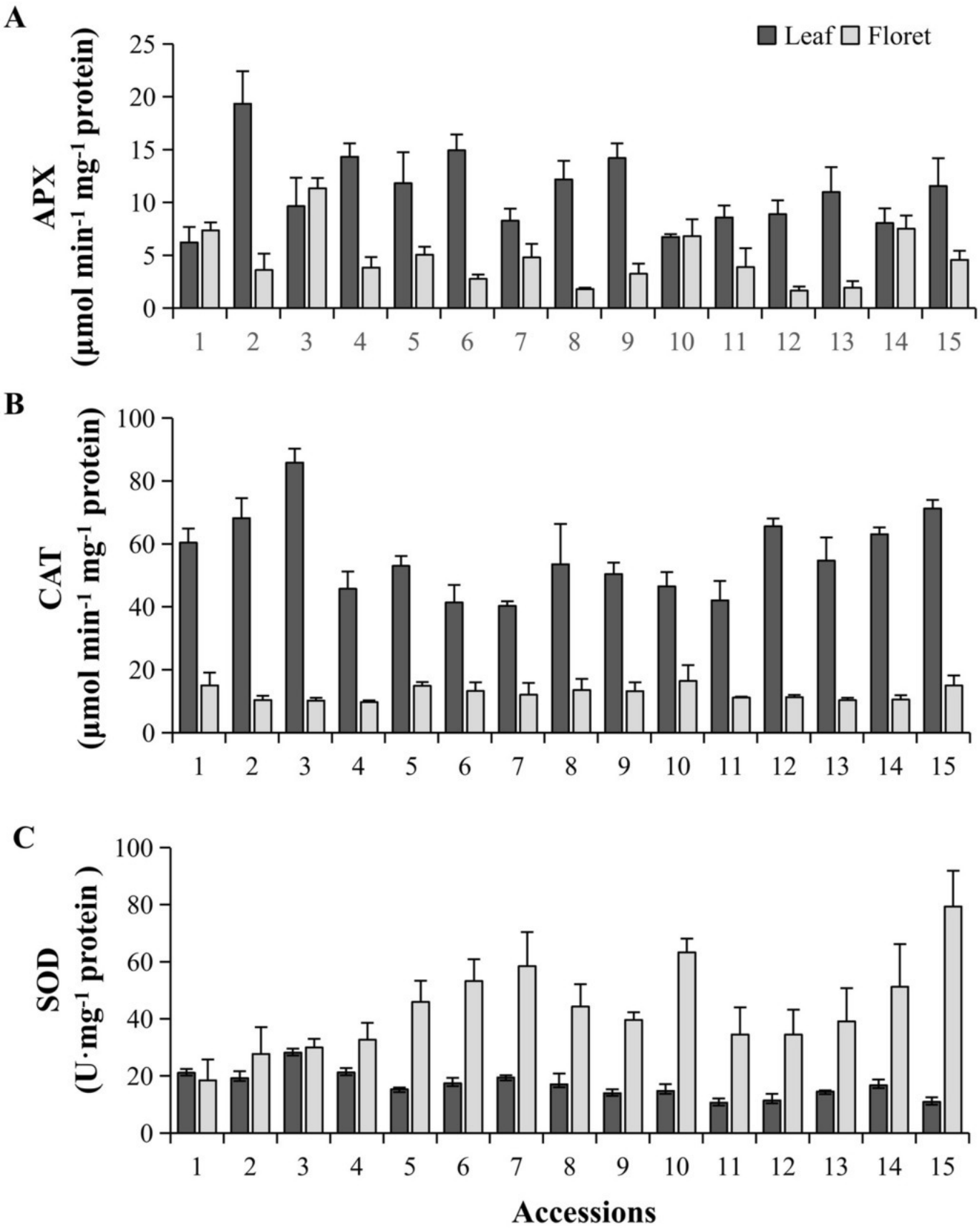

C
8
12

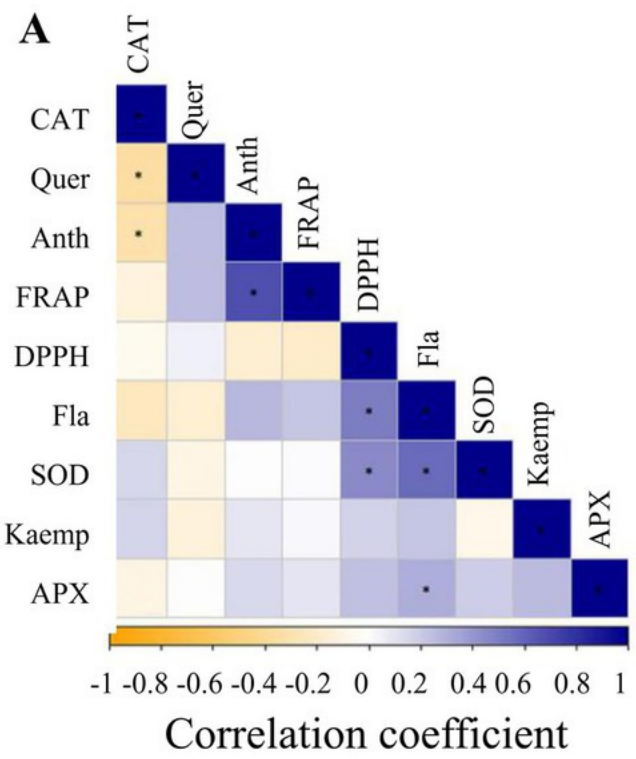

A
Phenylalanine+Acetyl-CoA
4-coumaroyl-CoA
3 malony-CoA
CHS
Naringenin chalcone
CHI
Naringenin
F3H
Dihydrokaempferol
Anthocyanins
F3'H
F3'5'H
Dihydroquercetin
Dihydromyrectin
FLS
FLS
FLS
Quercetin
Kaempferol
Myricetin

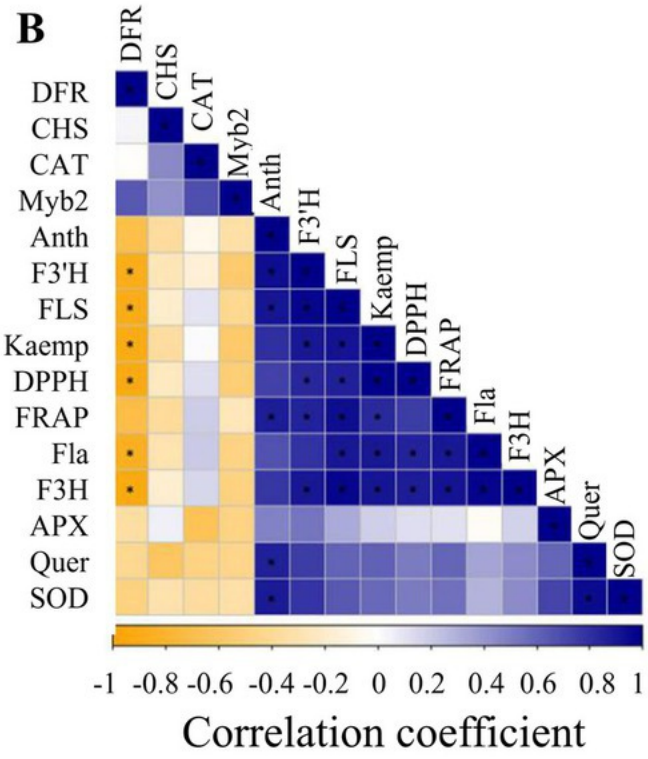

B
Relative expression
0
2
4
6
8
2
8
12
14
CHS
DFR
F3H
F3'H
FLS
Myb2
Genes

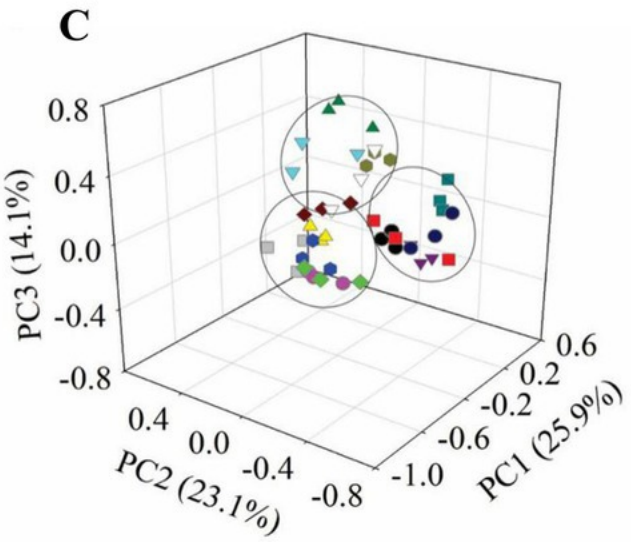

A
Kaempferol (µg g-1 FW)
160
120
80
40
0
Pacman
Diplomat
Marathon
*
Control
Se
Control
Se
Leaf
Floret
B
Quercetin (µg g-1 FW)
16
12
8
4
0
*
*
Control
Se
Control
Se
Leaf
Floret

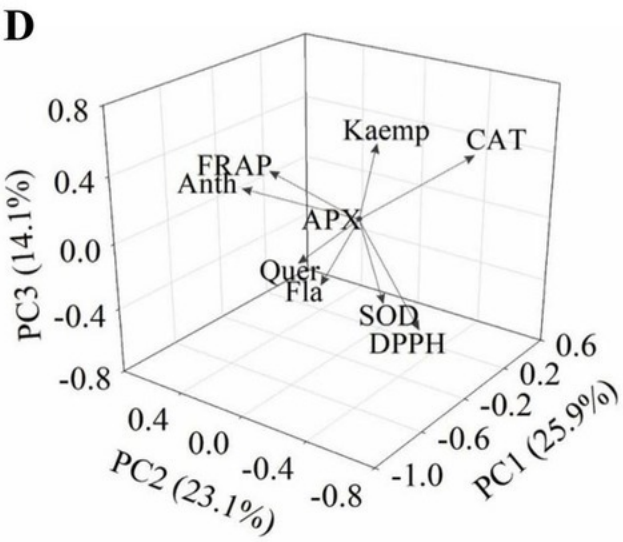

A
Flavonoids (µg RE g-1 FW)
400
320
240
160
80
0
Leaf
Floret
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
B
Athocyanins (µg g-1 FW)
4
3
2
1
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
C
DPPH (mg AEAC g-1 FW)
16
12
8
4
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
D
FRAP (µmol g-1 FW)
4
3
2
1
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Accessions


A
APX
(µmol min-1 mg-1 protein)
Leaf
Floret
25
20
15
10
5
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
B
CAT
(µmol min-1 mg-1 protein)
100
80
60
40
20
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
C
SOD
(U·mg-1 protein)
100
80
60
40
20
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Accessions


A
CAT
Quer
Anth
FRAP
DPPH
Fla
SOD
Kaemp
APX
-1 -0.8 -0.6 -0.4 -0.2 0 0.2 0.4 0.6 0.8 1
Correlation coefficient


B
DFR
CHS
CAT
Myb2
Anth
F3'H
FLS
Kaemp
DPPH
FRAP
Fla
F3H
APX
Quer
SOD
-1 -0.8 -0.6 -0.4 -0.2 0 0.2 0.4 0.6 0.8 1
Correlation coefficient


C
0.8
0.4
0.0
-0.4
-0.8
PC3 (14.1%)
0.4
0.0
-0.4
-0.8
PC2 (23.1%)
0.6
0.2
-0.2
-0.6
-1.0
PC1 (25.9%)


D
0.8
0.4
0.0
-0.4
-0.8
PC3 (14.1%)
Kaemp
CAT
FRAP
Anth
APX
Quer
Fla
SOD
DPPH
0.4
0.0
-0.4
-0.8
PC2 (23.1%)
0.6
0.2
-0.2
-0.6
-1.0
PC1 (25.9%)