COMPARATIVE STUDIES ON NUTRITIVE QUALITY AND ANTHOCYANIN SYNTHESIS OF PURPLE SELENIUM-ENRICHED WAXY CORN AND COMMON PURPLE WAXY CORN DURING KERNEL DEVELOPMENT

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Abstract. Anthocyanins and selenium (Se) have antioxidant properties and are crucial to human health. Previous works have well studied the role of exogenous Se fertilization in grain quality and anthocyanin formation in colored crops, whereas few reports on naturally Se-enriched colored grains exist. In this study, the quality, anthocyanin accumulation, and metabolism of natural Se-enriched purple waxy corn and common purple waxy corn were compared during kernel development. Natural Se-enriched purple waxy corn had higher concentrations of total Se, anthocyanins, flavones, starch (branched- and straight-chain), and sugar than common purple waxy corn, and the optimum eating date was 28 days after pollination. Genes related to anthocyanin biosynthesis were significantly upregulated in natural Se-enriched purple waxy corn, resulting in enriched anthocyanin metabolites (6-O-malonyl-beta-D-glu and 3-O-glu in Cy, Pn, and Pg). Most importantly, genetic alterations were observed which several genes and transcription factors were downregulated some branch pathways of lignin synthesis and accelerated anthocyanin synthesis. Our results profiled anthocyanin metabolism in naturally Se-enriched purple waxy corn to promote harvesting.
Keywords: *purple waxy corn, anthocyanin monomers, flavones, acylation, lignin synthesis*

Introduction

In human, selenium (Se) is a microelement element as it has antioxidant, anticancer, antibacterial, antiviral, and other biological functions (Muszyńska, 2020). Dietary Se deficiency is a global problem (Zhou et al., 2020). Globally, Se deficiency is associated with low Se concentrations in common cereal crops (Galić et al., 2021). Waxy corn (*Zea mays* L.), including yellow, red, and purple corn, is increasingly being consumed in China, Korea, Vietnam, Taiwan, Laos, Myanmar, and Thailand (Harakotr et al., 2014b; Aqil, 2020). In general, purple waxy corn has adequate nutrients, balanced amino acid composition, protein, fat, vitamin B group, vitamin C and minerals Fe, Mn, Cu, Zn, K, Ca, Se and other contents are higher than common corn, particularly anthocyanins in the pericarp. The anthocyanins about total concentration extent is 21 mg/100 g to 618 mg/100 g DW (Dry Weight) (Harakotr et al., 2014a; Nankar et al., 2016; Chatham et al., 2018; Li et al., 2019). Purple corn contains rich anthocyanins throughout the entire plant, such as cornflower pigments, peony pigments, geranium

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pigments, etc. (Chatham et al., 2018; Hong et al., 2020). Anthocyanins have antimutagenic, anticancer, antidiabetic, and anti-obesity activities, and can scavenge free radicals (Varga et al., 2013). Therefore, naturally purple, Se-enriched waxy corn is a popular functional food in modern cities.

Although waxy corn originated in China, it is consumed globally owing to its flavor characteristics and high nutritional value (Schwartz., 2009). However, there are relatively few natural Se-enriched waxy corn varieties, particularly the purple varieties. The purple waxy corn is preferred by consumers due to the health-promoting properties of the anthocyanin pigments in the aleurone or pericarp (Fukamachi et al., 2008). Jinnuo 8 is a fresh Se-enriched waxy corn variety bred in China. Its Se concentration meets the standard for Se-enriched corn (GB/T 22499-2008) and it contains high levels of anthocyanins. Purple waxy corn is harvested during the immature stage of grain development, which directly affects its market value (Mohamed., 2017). Therefore, it is necessary to define the fresh feeding period, that is, the physiological maturity period of the optimal food quality of seeds, from 20 to 30 days after pollination (Huang et al., 2019; Kim et al., 2020; Hong et al., 2020), progressive development of nutritional quality, and anthocyanin components throughout the fresh food period. The optimal period for nutritional quality has been determined.

Previous studies have mainly focused on the exogenous Se fertilization on grain quality and anthocyanin formation in colored crops, the physiological and biochemical mechanisms of anthocyanin synthesis and accumulation in common waxy corn, the effects of stress on the nutritional quality of waxy corn and anthocyanins, and the antioxidant activity of colored waxy corn at different maturation stages (Harakotr et al., 2014b; Hong et al., 2020; Kim et al., 2020; Guo et al., 2021). However, there is a lack of information on the nutritional quality and anthocyanin deposition of naturally Se-enriched purple waxy corn during the fresh-eating stage. This is related to the development and utilization of high-quality resources, such as Se-enriched purple waxy corn. Once this problem is resolved, it is expected to have important theoretical significance for the development and utilization of Se-enriched anthocyanin-rich purple waxy corn.

The specific objectives of the present study were to (1) explain the difference in the quality index (starch, sugar, Se, anthocyanins, and flavone) accumulation regularity in purple Se-enriched waxy corn and common purple waxy corn during the fresh feeding period, (2) distinguish the difference in anthocyanin components between purple Se-enriched waxy corn and common purple waxy corn, (3) evaluate gene expression variations between natural Se-enriched purple waxy corn and common purple waxy corn varieties using RNA-Seq data, and (4) select the best fresh eating period of natural Se-enriched purple waxy corn–Jinnuo 8. This study provides preliminary data on the differences between purple Se-enriched waxy corn and common purple waxy corn.

Materials and methods

Experimental site

From May in 2022 to October in 2022, this work was set out at locations in the Xiaoshentou Village, lvliang, Shanxi Province, China (37°59’ N, 111°41’ E, 1402 m above sea level). The region has a temperate continental climate with a mean annual temperature of 8.9°C, a mean annual precipitation of ~500 mm, and a frost-free period

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of ~154 days. The experimental field had cinnamon soil (Chinese soil taxonomy). The soil properties at 0–20 cm depth were 13.35 g kg^{-1} of soil organic matter (SOM), 1.87 g kg^{-1} of total nitrogen (N), 77.17 mg kg^{-1} of available nitrogen (N), and 5.79 mg kg^{-1}, available phosphorus (P), 135.27 mg kg^{-1} of exchangeable potassium (K), and 0.14 mg kg^{-1} of total Se.

Plant materials and harvest maturity

Two purple waxy corn cultivars, namely Jinnuo8 (E) and Jinnuo20 (CK), were planted. Seeds were provided by the Maize Research Institute of Shanxi Agricultural University (Shanxi Academy of Agricultural Sciences). Jinnuo8 is a naturally Se-enriched variety with a grain Se concentration of 0.473 mg kg^{-1}. Pre-plant broadcast manure at a dose of 30,000 kg ha^{-1} and basal fertilizer containing 180, 75, and 75 kg ha^{-1} of N, P_2O_5, and K_2O, respectively, were applied for field preparation. Weed control was performed by hand pulling at bolting. No additional water or fertilizer was applied during plant growth. Sowing dates was May 1, 2022, and plants was harvested on October 1, 2022.

Cobs were sampled in a range of increasing kernel maturities, measured from 20 to 30 d (days after pollination, 50% pollination). A sample of 5 cobs was harvested at 20 d, 22 d, 24 d, 26 d, 28 d, and 30 d. Three plants with roughly the same growth potential were taken for sampling, and then stored in a refrigerator at -80℃ for the determination of anthocyanin content and nutritional composition. Anthocyanin components were detected using kernels at 20 d, 24 d, and 28 d. Transcriptome analysis was conducted using Kernels at 28 d.

Determination of concentrations for assessing nutritive quality

The determination of Se content is carried out using the national standard method GB 5009.242-2007. Total Anthocyanin content (TAC) analysis was performed using the differential pH method (Siriwoharn et al., 2004). To measure flavone, corn kernels were weighed, 1.5 mL of 60% ethanol was added, and the seeds were extracted by shaking at 60℃ for 2 h, centrifuged at 25℃×12000 rpm for 10 min, and the supernatant was tested. The absorption value (D) was determined at 510 nm using a reagent blank as a control. The anthrone colorimetric method was used to determine the sugar concentration. Amylase and amylopectin levels were determined by the dual-wavelength method. The sum of amylase and amylopectin was used as the total starch content.

HPLC analysis of anthocyanin component

The standard anthocyanins (10 mg mallow pigment, cornflower pigment, geranium pigment, delphinium pigment, peony pigment, petunia pigment) were accurately weighed and dissolved in 100 mL of solvent (extraction solution: concentrated hydrochloric acid = 3:1 (V/V)) to prepare 100 μG/mL stock solution, and then diluted to 20, 40, 60, 80, and 100 μG/mL of working fluid respectively. After filtration with 0.45 μm filter membrane, Solution was used for high-performance liquid chromatography analysis. Chromatographic detection conditions were as below: mobile phase A was 0.5% formic acid, mobile phase B was acetonitrile, A: B = 90:10 (V/V), and flow rate was 0.8 mL/min. The detection wavelength was 520 nm, and the injection amount was 10 μL. The temperature of the column box was 32℃.

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RNA-sequencing and analysis

Total RNA from corn seeds was extracted according to the operating procedures of the polysaccharide polyphenol plant total RNA extraction kit (Bio Teke, Beijing, China). RNA purity (OD260/280, OD260/230) was detected using Nanodrop 2000 (Thermo Fisher, USA), and RNA fragment length was detected using Agilent 2100 (Agilent, USA). After passing the RNA detection, it is used for constructing the cDNA library. The high-quality library was sequenced using the Illumina Hi SeqTM 2000. The raw data was filters using Fastp (v0.19.3) software to obtain Clean reads. Then Trinity (v2.4.0) was used to assemble the transcripts of the obtained Clean reads. Finally Corset was used to cluster and de redundant the assembled transcripts to obtain unigenes. DEseq software (1.10.1) was used to screen differentially expressed genes, with a screening criterion of FDR (false discovery rate) < 0.05 and log2 | fold change, FC | ≥ 1. Based on the KEGG database, KOBAS software (v3.0) was used to enrich metabolic pathways of significantly differentially expressed genes (FDR < 0.05).

Quantitative real-time PCR assay

Total RNA was extracted from the seed samples of Jinnuo 8 (E) and Jinnuo 20 (CK) 28 d after pollination using TRIzol reagent. The RNA was reverse transcribed into cDNA using High Capacity cDNA reverse transcription kit, and then SYBR was used ™ Green PCR Premix and Step One Plus ™ Real time fluorescence quantitative PCR system for real-time fluorescence quantitative PCR. The relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Ren et al., 2022).

Statistical analysis

All data were analyzed using SPSS software (version 17.0; China) and are expressed as the mean ± standard deviation (SD) of biological triplicates. SPSS software was used for the analysis of variance. Least Significant Difference (LSD) tests at $P = 0.05$ was used for comparison of means.

Results and discussion

Nutritional quality

With increasing kernel maturity from 20 d to 30 d, Se, total anthocyanin, total flavone, and starch (branched- and straight-chain) concentrations increased significantly ($P < 0.05$), whereas total sugar showed an increasing trend followed by a decreasing trend (*Fig. 1*). First, the Se concentrations of purple Se-enriched waxy corn had reached the standard of Se-enrich cereals (0.15 mg kg^{-1} ~ 0.30 mg kg^{-1}) 20 d after pollination, the range significantly increased from 0.23 to 0.31 mg kg^{-1}, and the overall Se concentration was about twice that of common purple waxy corn (*Fig. 1a*). This may be related to their anthocyanin composition; anthocyanins and Se can influence each other (Pu et al., 2021). A similar result has been reported previously, where the Se concentration may be due to the tendency towards enrichment with the accumulation of pigment in the ripening stage of colored-grain wheat (Xia et al., 2023).

In previous investigations of many crops, the TAC was found to be 138.1 mg 100 g^{-1} in black -colored waxy corn (Hu and Xu, 2011). When total anthocyanin concentration (TAC) was measured, the concentration of TAC (ranging from 78.24 to 208.49 mg $^{-1}$ of

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fresh weight (FW) in all edible stages) in waxy corn in this study were higher than those reported in a previous study, and that of the purple Se-enriched waxy corn and common purple waxy corn reached a maximum of 208.49 mg 100 g^{-1} FW and 145.21 mg 100 g^{-1} FW at 30 d, respectively. However, the growth rate of the two varieties increased after decreasing, reaching a maximum at 28 d when purple Se-enriched waxy corn was 13.5% and common purple waxy corn was 14.5%. Although TAC accumulation continued to slightly beyond 28 d, this may be due partly to moisture loss from the kernels, causing the growth rate to slow (*Fig. 1b*). This result was similar to maize and purple sweet corn of other studies (Li et al., 2019; Hong et al., 2020). Therefore, the difference between the two varieties of anthocyanins is caused by the plant genotype or differences in Se in the crops. The reasons for this need to be further investigated.

Anthocyanins are a subclass of flavonoids (Lepiniec et al., 2006). In this study, significant differences ($P < 0.05$) among corn genotypes were found for total flavonoid concentration (TFC) at the edible stage. Purple Se-enriched waxy corn had a higher TFC than common purple waxy corn. With increasing kernel maturity from 20 d to 30 d, the growth rate of purple Se-enriched waxy corn increased and reached its maximum value (38.1%) at 30 d (*Fig. 1c*). Six flavonoids were detected in immature grains (*Table 1*). In contrast to Se, TAC, and TFC, the sugar content first increased and then decreased in both corn types. The sugar content reached its maximum of 196.33 mg g^{-1} (CK) and 235.94 mg g^{-1} (E) at 28 d, respectively (*Fig. 1d*). The purple Se-enriched waxy corn starch content was higher than that of common purple waxy corn (*Fig. 1e, f*). During ripening, starch content continued to increase. Because the amylopectin content in waxy corn accounted for a large proportion, the sugar content in the subsequent grains decreased, which was also the reason attributed to the strong waxy corn. Based on the above comprehensive indicators, the optimal eating stage for purple waxy corn would be at a harvest maturity of 28 d for purple waxy corn, because at this time the concentrations of anthocyanin, Se, and sugar were high. Although the concentration of anthocyanin and Se in the later stage accumulates to a value greater than that at the edible stage, the starch content will continue to increase, and the sugar content will decrease, which affectsthus affecting the taste of people when eating. These results are consistent with those previously reported for purple-pericarp corn (O'Hare et al., 2015).

Identification and quantification of anthocyanins

The higher concentrations were total Se and anthocyanin in purple Se-enriched waxy corn during kernel development (*Fig. 1a, b*). Since the difference in data collected every two days was not significant, we chose a treatment every four days (22 d, 24 d and 28 d) for the determination of anthocyanin components. Thirty-nine anthocyanin derivatives (monomers) extracted from the two purple-waxy corn cultivars fallen in eight anthocyanin classes. Nine Cy isomers, six Pn isomers, eight Pg isomers, six flavonoid isomers, four Pt isomers, three Dp isomers, two Mv isomers, and one Procyanidin B3 were traced (*Table 1*). The eight categories were as follows: Cy > Pn > Pg > Flavonoid > Pt > Dp > Mv > Procyanidin B3. Pt, Dp, Mv, and Procyanidin B3 were negligible minor components in most plant tissues, and Paulsmeyer et al. (2022) reported that Pn is a minor component in most plant tissues. The content of anthocyanins in Se-enriched purple waxy corn was higher than that in common purple waxy corn (except Procyanidin B3) (*Fig. 2*), which might be one of the reasons for the difference in the synergistic effect of Se content and anthocyanins in grains, which needs to be further verified.

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Table 1. *Mass spectrometry data and identification of anthocyanins from purple waxy corn*

Peaks	Individual anthocyanins	Class	Abbreviation	[M+]
1	Cyanidin-3,5-O-diglucoside	Cyanidin	Cy-3,5-O-diglu	611
2	Cyanidin-3-O-xyloside	Cyanidin	Cy-3-O-xyl	419
3	Cyanidin-3-O-rutinoside	Cyanidin	Cy-3-O-rut	595
4	Cyanidin-3-O-(6-O-p-coumaroyl)-glucoside	Cyanidin	Cy-3-O-(6-O-coumaroyl)-glu	595
5	Cyanidin-3-O-sophoroside	Cyanidin	Cy-3-O-sop	611
6	Cyanidin-3-O-glucoside	Cyanidin	Cy-3-O-glu	449
7	Cyanidin-3-O-galactoside	Cyanidin	Cy-3-O-gal	449
8	Cyanidin-3-O-arabinoside	Cyanidin	Cy-3-O-ara	419
9	Cyanidin-3-O-(6-O-malonyl-beta-D-glucoside)	Cyanidin	Cy-3-O-(6-O-malonyl-beta-D-glu)	535
10	Delphinidin-3-O-glucoside	Delphinidin	Dp-3-O-glu	465
11	Delphinidin-3-O-rutinoside	Delphinidin	Dp-3-O-rut	611
12	Delphinidin-3-O-rutinoside-5-O-glucoside	Delphinidin	Dp-3-O-rut-5-O-glu	773
13	Malvidin-3-O-5-O-(6-O-coumaroyl)-diglucoside	Malvidin	Mv-3-O-5-O-(6-O-coumaroyl)-diglu	801
14	Malvidin-3-O-glucoside	Malvidin	Mv-3-O-glu	493
15	Pelargonidin-3-O-arabinoside	Pelargonidin	Pg-3-O-ara	403
16	Pelargonidin-3-O-rutinoside	Pelargonidin	Pg-3-O-rut	579
17	Pelargonidin-3-O-glucoside	Pelargonidin	Pg-3-O-glu	433
18	Pelargonidin-3-O-5-O-(6-O-coumaroyl)-diglucoside	Pelargonidin	Pg-3-O-5-O-(6-O-coumaroyl)-diglu	741
19	Pelargonidin-3-O-(6-O-malonyl-beta-D-glucoside)	Pelargonidin	Pg-3-O-(6-O-malonyl-beta-D-glu)	519
20	Pelargonidin-3-O-(6-O-p-coumaroyl)-glucoside	Pelargonidin	Pg-3-O-(6-O-p-coumaroyl)-glu	579
21	Pelargonidin-3-O-sophoroside	Pelargonidin	Pg-3-O-sop	595
22	Pelargonidin-3,5-O-diglucoside	Pelargonidin	Pg-3,5-O-diglu	595
23	Peonidin-3-O-(6-O-malonyl-beta-D-glucoside)	Peonidin	Pn-3-O-(6-O-malonyl-beta-D-glu)	549
24	Peonidin-3-O-(6-O-p-coumaroyl)-glucoside	Peonidin	Pn-3-O-(6-O-p-coumaroyl)-glu	609
25	Peonidin-3,5-O-diglucoside	Peonidin	Pn-3,5-O-diglu	625
26	Peonidin-3-O-arabinoside	Peonidin	Pn-3-O-ara	433
27	Peonidin-3-O-glucoside	Peonidin	Pn-3-O-glu	463
28	Peonidin-3-O-rutinoside	Peonidin	Pn-3-O-rut	609
29	Petunidin-3-O-rutinoside	Petunidin	Pt-3-O-rut	625
30	Petunidin-3-O-glucoside	Petunidin	Pt-3-O-glu	479
31	Petunidin-3-O-(6-O-p-coumaroyl)-glucoside	Petunidin	Pt-3-O-(6-O-p-coumaroyl)-glu	625
32	Petunidin-3-O-(6-O-malonyl-beta-D-glucoside)	Petunidin	Pt-3-O-(6-O-malonyl-beta-D-glu)	565
33	Procyanidin B3	Procyanidin		578
34	Dihydrokaempferol	Flavonoid	Dihydrokaempferol	288
35	Naringenin-7-O-glucoside	Flavonoid	Naringenin-7-O-glu	434
36	Quercetin-3-O-glucoside	Flavonoid	Quercetin-3-O-glu	464
37	Rutin	Flavonoid	Rutin	610
38	Naringenin	Flavonoid	Naringenin	272
39	Kaempferol-3-O-rutinoside	Flavonoid	Kaempferol-3-O-rut	594

Anthocyanin components increased with kernel maturity but the number of anthocyanin components in common purple waxy corn was lower than that in Se-enriched waxy corn. Interestingly, although kernel maturity had a significant effect on total anthocyanin accumulation (*Fig. 1*), the proportion of individual anthocyanins changed only slightly with increasing maturity (*Table 2*). The main significant changes observed were the concentration of Cy, Pn, and Pg, the major constituents, representing 44.5%~61.3%, 15.9% ~19.9%, and 13.8%~17.2% of the total anthocyanin content (TAC), respectively. These results are consistent with those of previous studies on purple corn, purple waxy corn, and purple pericarp sweet corn (Aoki et al., 2002; García-Tejeda et al., 2015; Kim et al., 2020; Hong et al., 2021).

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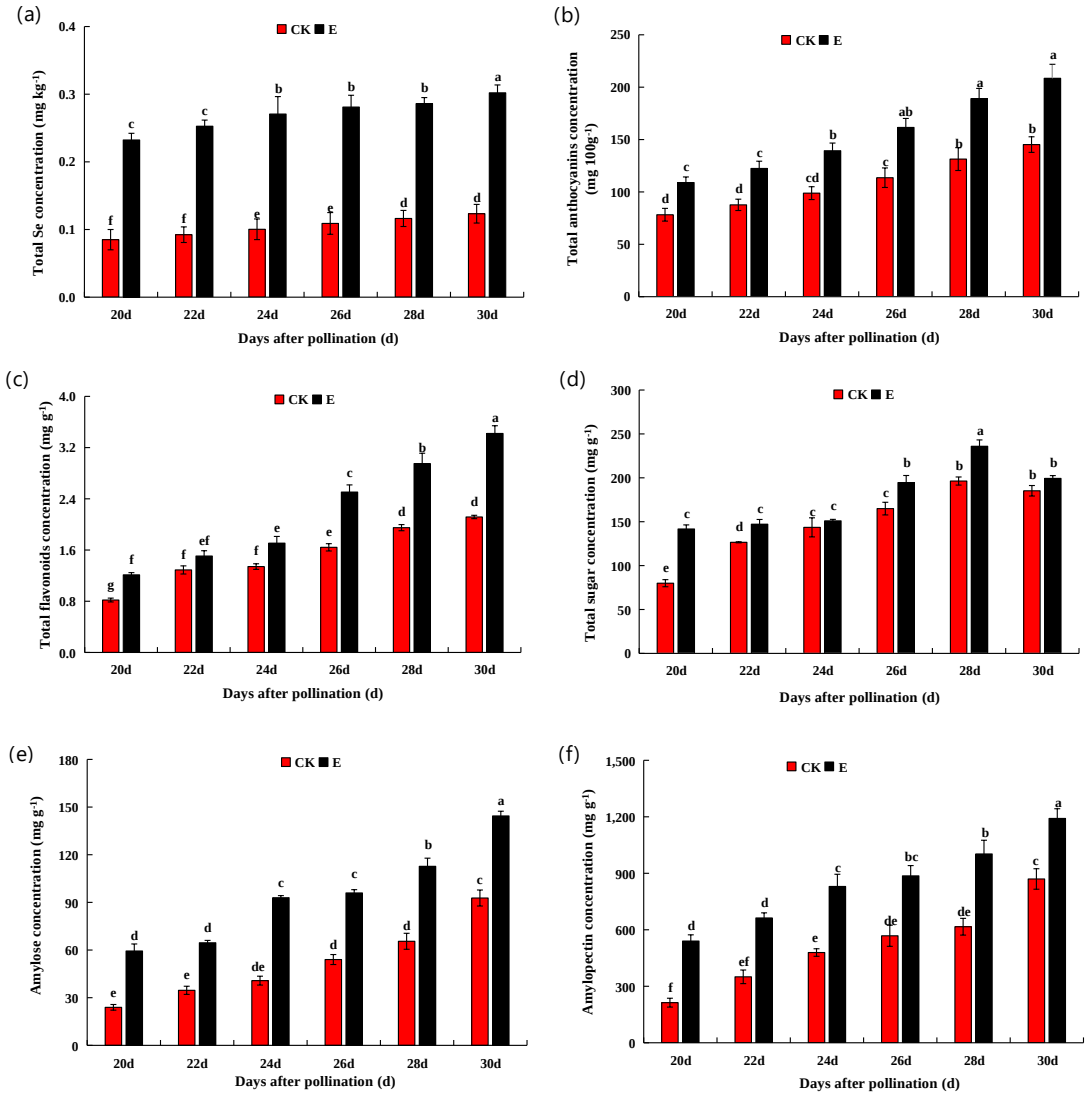

Figure 1. *The concentration of total Se, anthocyanins, flavonoids, sugar and starch concentrations of two purple waxy-corn cultivars during kernel development. CK, Jinnuo20; E, Jinnuo8; d, days after pollination; Error bars indicate ± standard error (SE). The means are not significantly different when followed by the same lowercase letter using LSD at* $P < 0.05$

Most anthocyanins identified in this study have been previously described in corn. The most abundant compounds identified were glucoside, 6-O-malonyl-beta-D-glu, and the 3-O-glu forms of Cy, Pn, and Pg. In 9 Cy isomers, Cy-3-O-(6-O-malonyl-beta-D-glu) accounted for 31.9%~45.3% of TAC followed by Cy-3-O-glu (11.8%~16.8%). In 6 Pn isomers, Pn-3-O-(6-O-malonyl-beta-D-glu) accounted for 12.6%~16.1% of TAC followed by Pn-3-O-glu (1.6%~2.6%). In 8 Pg isomers, Pg-3-O-(6-O-malonyl-beta-D-glu) accounted for 10.6%~13.5% of TAC followed by Pg-3-O-glu (1.5%~3.2%). Here, flavonoid components were also detected Quercetin-3-O-glu and Rutin accounted for 0.95%~1.50% and 2.04%~3.28% of TAC, respectively (*Fig. 2; Table 2*). The proportions of other anthocyanin components were very small and could be ignored (*Fig. 2; Table 3*). One striking finding of this study was the high proportion of 6-O-

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malonyl-beta-D-glu, and 3-O-glu forms of Cy, Pn, and Pg in Se-enriched waxy corn; however, it is uncertain why the concentration of 6-O-malonyl-beta-D-glu, and 3-O-glu forms increased. It is possible that acylation is an important modification of anthocyanins, as these groups interact with stability and increase the total anthocyanin content in corn (Zhao et al., 2017; Paulsmeyer et al., 2018) and purple-colored leaf tea (Shi et al., 2021). Another reason may be that Se promotes the synthesis of acylated anthocyanin monomers (Xia et al., 2023). Many studies have investigated the regulation of anthocyanin synthesis in crops by exogenous Se (Pu et al., 2021; Zhang et al., 2022; Xia et al., 2023). However, to the best of our knowledge, this is the first report of pigment accumulation in natural Se-enriched waxy corn during the edible stage.

Table 2. *The concentration of Cyanidin (Cy), Peonidin (Pn), Pelargonidin (Pg) and Flavonoid components in two purple waxy corn (mg 100 g^{-1})*

Cyanidin (Cy), Peonidin (Pn) and Pelargonidin (Pg) components	CK			E		
	20 d	24 d	28 d	20 d	24 d	28 d
Cy-3,5-O-diglu	0.033 c	0.055 c	0.100 b	0.056 c	0.124 b	0.152 a
Cy-3-O-xyl	0.206 a	0.196 a	0.187 a	0.162 a	0.076 b	0.062 b
Cy-3-O-rut	0.322 d	0.387 d	0.440 d	1.049 c	1.499 b	2.045 a
Cy-3-O-(6-O-coumaroyl)-glu	0.001 c	0.003 a	0.003 a	—	—	0.002 b
Cy-3-O-sop	0.030 c	0.061 b	0.065 b	0.027 c	0.070 b	0.088 a
Cy-3-O-glu	9.228 e	13.173 d	18.562 c	18.323 c	22.614 b	28.146 a
Cy-3-O-gal	0.118 b	0.191 a	0.229 a	0.076 b	0.103 b	0.201 a
Cy-3-O-ara	0.024 b	0.029 b	0.040 a	0.008 c	0.012 c	0.023 b
Cy-3-O-(6-O-malonyl-beta-D-glu)	24.986 d	44.816 c	47.750 c	47.108 c	57.215 b	67.029 a
Pn-3-O-(6-O-malonyl-beta-D-glu)	12.622 b	14.647 b	20.684 a	15.359 b	21.757 a	23.786 a
Pn-3-O-(6-O-p-coumaroyl)-glu	0.005 b	0.006 b	0.011 a	—	—	0.001 c
Pn-3,5-O-diglu	0.025 b	0.028 b	0.032 b	0.022 b	0.062 a	0.065 a
Pn-3-O-ara	0.006 b	0.007 b	0.008 b	0.009 b	0.014 a	0.017 a
Pn-3-O-glu	1.953 b	1.922 b	2.068 b	1.884 b	3.626 a	3.757 a
Pn-3-O-rut	0.291 c	0.494 c	0.531 c	0.980 b	2.199 a	2.435 a
Pg-3-O-ara	0.004 b	0.006 b	0.007 b	0.006 b	0.007 b	0.016 a
Pg-3-O-rut	0.081 d	0.227 b	0.333 a	0.051 d	0.102 c	0.106 c
Pg-3-O-glu	1.755 b	3.118 a	3.438 a	2.031 b	2.091 b	3.823 a
Pg-3-O-5-O-(6-O-coumaroyl)-diglu	0.001 c	0.001 c	0.001 c	0.001 c	0.002 b	0.003 a
Pg-3-O-(6-O-malonyl-beta-D-glu)	10.597 c	13.315 b	14.125 b	12.582 b	15.184 b	20.005 a
Pg-3-O-(6-O-p-coumaroyl)-glu	—	—	—	0.002 b	0.002 b	0.004 a
Pg-3-O-sop	0.026 c	0.051 b	0.059 b	0.044 b	0.046 b	0.122 a
Pg-3,5-O-diglu	0.008 c	0.017 b	0.018 b	0.011 c	0.014 c	0.023 a
Dihydrokaempferol	0.013 d	0.043 c	0.068 b	0.031 c	0.071 b	0.105 a
Kaempferol-3-O-rut	0.693 d	0.870 c	0.929 c	1.795 b	1.960 a	1.904 a
Quercetin-3-O-glu	1.085 c	1.125 c	1.250 bc	1.620 b	2.087 a	2.446 a
Rutin	1.928 f	2.316 e	2.685 d	3.576 c	5.076 b	5.619 a
Naringenin	0.007 e	0.014 c	0.014 c	0.012 d	0.037 b	0.042 a
Naringenin-7-O-glu	0.072 d	0.081 d	0.096 c	0.118 c	0.151 b	0.182 a

Different lowercase letters (a, b) indicated significant differences among different treatment of the same line at $P < 0.05$

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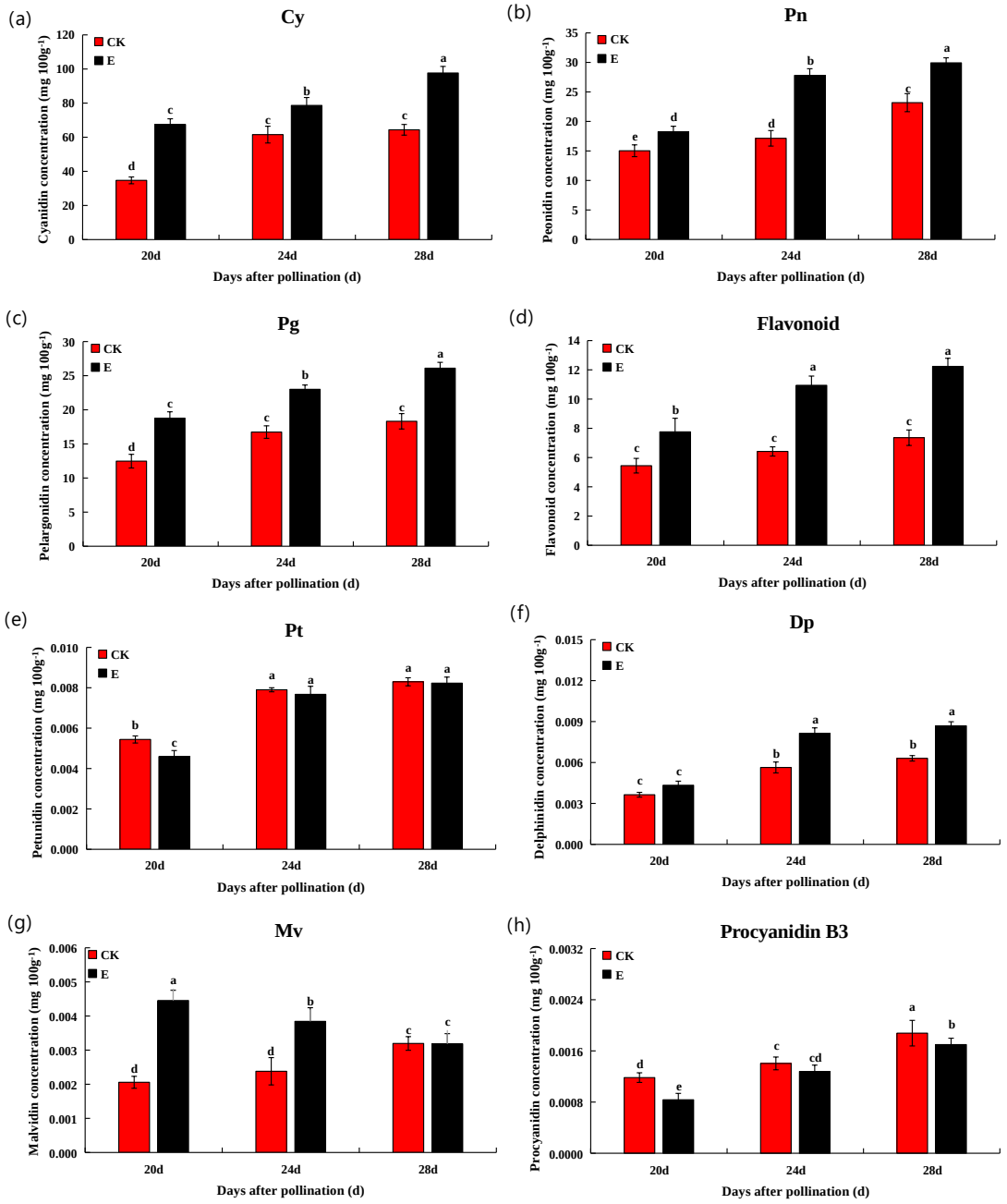

Figure 2. *The concentration of anthocyanin component of two purple waxy-corn cultivars during kernel development. CK, Jinnuo20; E, Jinnuo8; d, days after pollination; Cy, Cyanidin; Dp, Delphinidin; Mv, Malvidin; Pg, Pelargonidin; Pn, Peonidin; Pt, Petunidin; Error bars indicate ± standard error (SE). The means are not significantly different when followed by the same lowercase letter using LSD at P < 0.05*

Functional analysis of genes and experimental validation

We further investigated the changes in the gene expression profiles of the two purple waxy corn samples, 28 d after pollination. These two genotypes exhibited significant differentially expressed genes (DEGs). In *Figure 3a*, between the Jinnuo20 (CK) and Jinnuo8 (E), we had 8125 (5158 up-regulated and 2967 down-regulated) DEGs. KEGG

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analysis was performed to investigate the functions of the genes (*Fig. 3b*). KEGG-enriched pathways were classified into three parts: metabolism (16 paths), environmental information processing (three paths), and organismal systems (one path). In terms of metabolism, the most enriched KEGG terms contributing to the DEGs were phenylpropanoid biosynthesis, starch and sucrose metabolism, flavonoid biosynthesis, and zeatin biosynthesis. To obtain more biological information on the anthocyanin accumulation mechanism in purple Se-enriched waxy corn, the KEGG enrichment pathways were analyzed (*Table 4*). The pathway related to anthocyanin biosynthesis and phenylpropanoid biosynthesis (zma00940) ranked first (37 upregulated and 27 downregulated). Flavonoid biosynthesis (zma00941) ranked third (eight upregulated and four downregulated), mainly including *PAL*, *C4H*, *CHS*, *F3H*, *DFR*, *ANS*, *4CL*, *F5H*, and *COMT*, which were identified in both corn varieties. Genes for synthesizing grain anthocyanins were significantly up-regulated (*PAL*, *C4H*, *CHS*, *F3H*, *DFR*, and *ANS*) and those for synthesizing lignin (*4CL*, *F5H*, and *COMT*) were significantly downregulated in purple Se-enriched waxy corn. This result is in accordance with those of other studies on Alfalfa, in which Se application increased flavonoid content, strengthened substrate diversion, and subsequently reduced the level of substrate used in the lignin metabolic pathway, thereby reducing lignin deposition (Zhang et al., 2023).

Table 3. *The concentration of Petunidin (Pt), Delphinidin (Dp), (Mv) and Procyanidin B3 components in two purple waxy corn (mg 100 g^{-1})*

Petunidin (Pt), Delphinidin (Dp), Malvidin (Mv) and Procyanidin B3 components	CK			E		
	20 d	24 d	28 d	20 d	24 d	28 d
Pt-3-O-rut	—	0.002 a	0.002 a	0.001 b	0.001 b	0.001 b
Pt-3-O-glu	0.002 b	0.002 b	0.003 a	0.002 b	0.002 b	0.003 a
Pt-3-O-(6-O-p-coumaroyl)-glu	0.002 b	0.002 b	0.003 a	0.001 c	0.001 c	0.003 a
Pt-3-O-(6-O-malonyl-beta-D-glu)	0.001 c	0.002 b	0.003 a	0.002 b	0.002 b	0.002 b
Dp-3-O-glu	0.001 c	0.002 b	0.003 a	0.001 c	0.001 c	0.002 b
Dp-3-O-rut	0.001 b	0.001 b	0.002 a	0.001 b	0.001 b	0.001 b
Dp-3-O-rut-5-O-glu	0.001 c	0.005 a	0.005 a	0.003 b	0.003 b	0.003 b
Mv-3-O-5-O-(6-O-coumaroyl)-diglu	—	—	—	—	—	—
Mv-3-O-glu	0.001 b	0.002 a	0.002 a	0.001 b	0.001 b	0.001 b
Procyanidin B3	0.001 c	0.002 b	0.003 a	0.002 b	0.002 b	0.003 a

Different lowercase letters (a, b) indicated significant differences among different treatment of the same line at $P < 0.05$

PAL is the first enzyme in the biosynthesis of anthocyanins, and the expression of two coding genes (*LOC100273579* and *LOC100381820*) was upregulated, suggesting that the accumulation of 4-coumaric-CoA could provide more substrates for anthocyanin synthesis (Kong, 2015; Wang et al., 2017). *CHS* is a key enzyme that regulates the conversion of 4-coumaric-CoA to naringenin through the anthocyanin biosynthesis pathway (Yuan et al., 2022); one coding gene (*LOC100282642*) was upregulated and it produces chalcone. In addition, the upregulated expression of the *DFR* gene (*LOC100272982*) allowed more dihydrokaempferol to enter the anthocyanin synthesis pathway, thus producing more colorless anthocyanins. *ANS* is the final key enzyme involved in anthocyanin synthesis. In the present study, the expression of one

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gene encoding *ANS* (*LOC100127010*) was up-regulated, which directly affected anthocyanin accumulation (Reddy et al., 2007). Intermediates of the anthocyanin biosynthetic pathway can also be used to synthesize other secondary substances. *4CL* is the primary precursor of lignin and alkaloids (Knobloch and Hahlbrock, 1975; Leple et al., 2007). In this study, we found that the gene encoding *4CL* (*LOC542166*) in purple Se-enriched waxy corn was significantly downregulated, which reduced the levels of intermediates of the lignin synthesis pathway. Simultaneously, the gene encoding caffeic acid, *COMT* (*LOC100125646*), and the gene encoding *F5H* (*LOC103643375*) were significantly downregulated. *COMT* and *F5H* play catalytic roles in multiple processes of lignin synthesis (Humphreys et al., 1999; Tu et al., 2010), and downregulation of the genes encoding these two enzymes reduces lignin accumulation, allowing more substrates to enter the anthocyanin pathway. In summary, during lignin synthesis, genes encoding related enzymes were significantly downregulated, resulting in increased substrate flow for anthocyanin synthesis. This work concurs with that of a recent study on Se metabolism in color-grained wheat (Xia et al., 2023). However, in this study, regardless of the anthocyanin or lignin pathways, the related gene changes were not as significant as those published by the research group on exogenous Se enrichment of color-grained wheat, which may be due to the strong effect of exogenous Se enrichment on crops. However, this phenomenon also occurred in naturally Se-enriched corn, indicating that the changes in anthocyanins shown in *Figure 1* were related to Se enrichment.

Table 4. *The significantly enriched KEGG pathways in two purple waxy corn*

Pathway ID	Pathway	Up	Down	*P*-value
zma00940	Phenylpropanoid biosynthesis	37	27	0.000000067
zma00500	Starch and sucrose metabolism	35	28	0.000000111
zma04075	Plant hormone signal transduction	74	27	0.000009752
zma00941	Flavonoid biosynthesis	8	4	0.000300719
zma00908	Zeatin biosynthesis	14	4	0.000357892
zma00051	Fructose and mannose metabolism	12	15	0.000418679
zma04626	Plant-pathogen interaction	43	27	0.000485675
zma00592	alpha-Linolenic acid metabolism	16	5	0.000715522
zma00480	Glutathione metabolism	33	6	0.000736156
zma00052	Galactose metabolism	16	9	0.000937205
zma04016	MAPK signaling pathway - plant	35	18	0.001324915
zma00520	Amino sugar and nucleotide sugar metabolism	21	30	0.001548829
zma00906	Carotenoid biosynthesis	9	8	0.002950569
zma00904	Diterpenoid biosynthesis	12	2	0.003164079
zma00062	Fatty acid elongation	8	8	0.005948454
zma02010	ABC transporters	7	3	0.009914099
zma00591	Linoleic acid metabolism	7	1	0.011960383
zma00902	Monoterpenoid biosynthesis	6	1	0.012607753
zma00010	Glycolysis/gluconeogenesis	21	23	0.013417571
zma00910	Nitrogen metabolism	10	5	0.014734846

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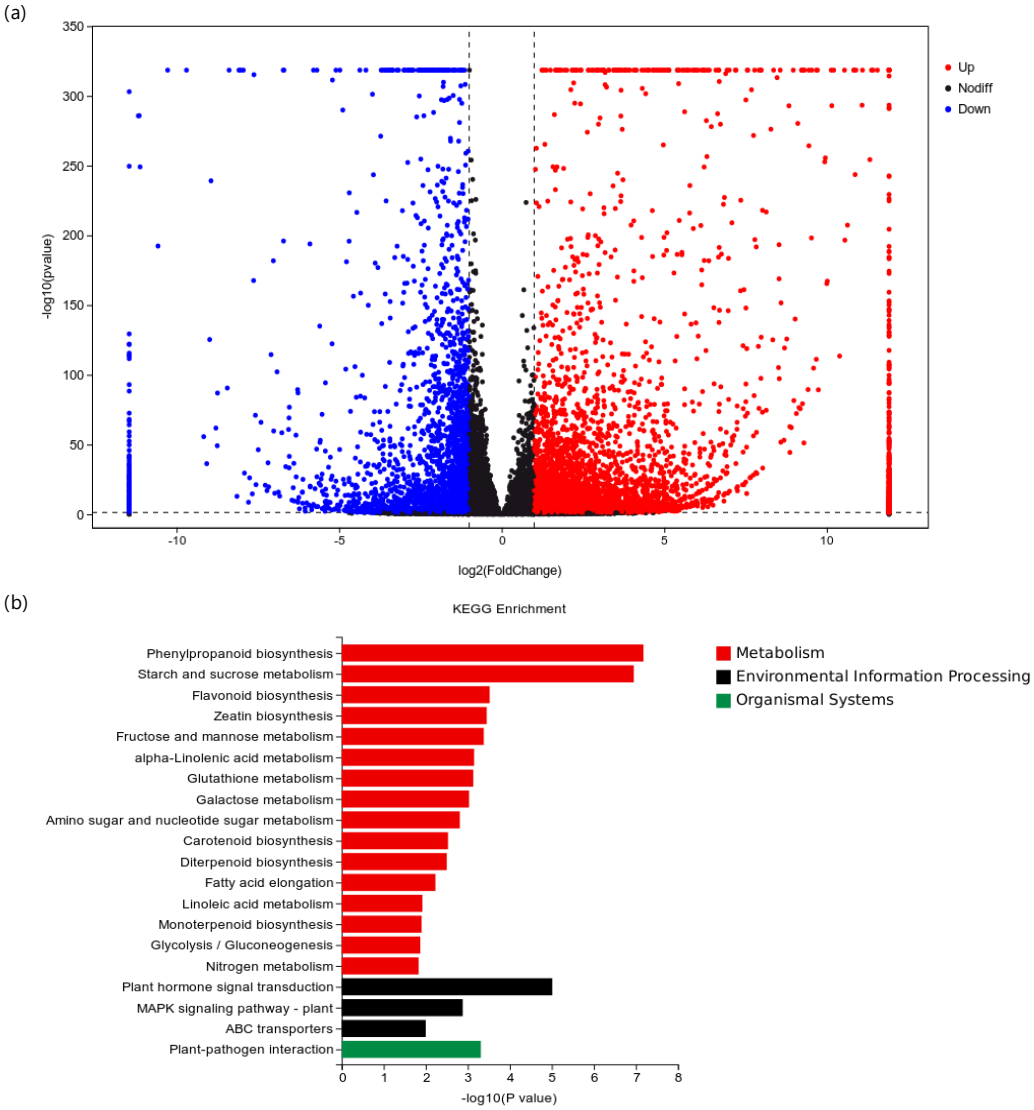

Figure 3. *Preliminary analysis of transcriptomics data at the 28 day after pollination. (a) Volcano plots of DEGs between CK and E, Red dots represent up regulation and blue dots shows down regulations; (b) Significantly enriched KEGG pathways between CK and E, the y-axis represents KEGG pathways; the x-axis indicates the enrichment score*

Anthocyanin synthesis is directly regulated by key enzymes such as *PAL*, *CHS*, *DFR*, and *ANS*. However, their gene expression levels are regulated by transcription factors, such as *R2R3-MYB*, *bHLH*, *WD40*, *bZIP*, *MADS*, and *WRKY* (Hichri et al., 2011; Lu et al., 2015). A total of 518 transcription factor genes were identified from DEGs, which could be divided into 19 families (*Fig. 4a*), including 83 MYB (16%), 63 *bHLH* (12%), 58 *NAC* (11%), 43 *VOZ* (8%), 32 *WRKY* (6%), 28 *ERF* (6%), 26 *C2H2* (5%), 24 *bZIP* (5%), 22 *HB-other* (4%), 20 *G2-like* (4%), 17 *ARF* (3%), 16 *ZF-HD* (3%), 15 *TCP* (3%), 14 *TALE* (3%), 13 *Trihelix* (3%), 12 *HD-ZIP* (2%), 11 *C3H* (2%), 11 *GRAS* (2%), and 10 *NF-YB* (2%). Among these transcription factor family members, *MYB* occupied the largest proportion, followed by *bHLH* and *NAC*. Notably, 72.8% of the transcription factor genes were significantly upregulated and only 27.2% were significantly downregulated, indicating that most of the differentially expressed transcription factor

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genes in purple Se-enriched waxy corn grains were upregulated (*Fig. 4b*). There were more upregulated genes than downregulated genes in the following families: *MYB* (57 upregulated and 27 downregulated), *bHLH* (63 upregulated and 0 downregulated), *NAC* (41 upregulated and 17 downregulated), *VOZ* (25 upregulated and 18 downregulated), *WRKY* (25 upregulated and 7 downregulated), and *ERF* (20 upregulated and 8 downregulated) (*Fig. 4b*). This is consistent with previous studies on G. biloba (Meng et al., 2019) and wheat (Pu et al., 2021). These results suggested that the transcription factors may be involved in the regulation of Se-induced gene expression.

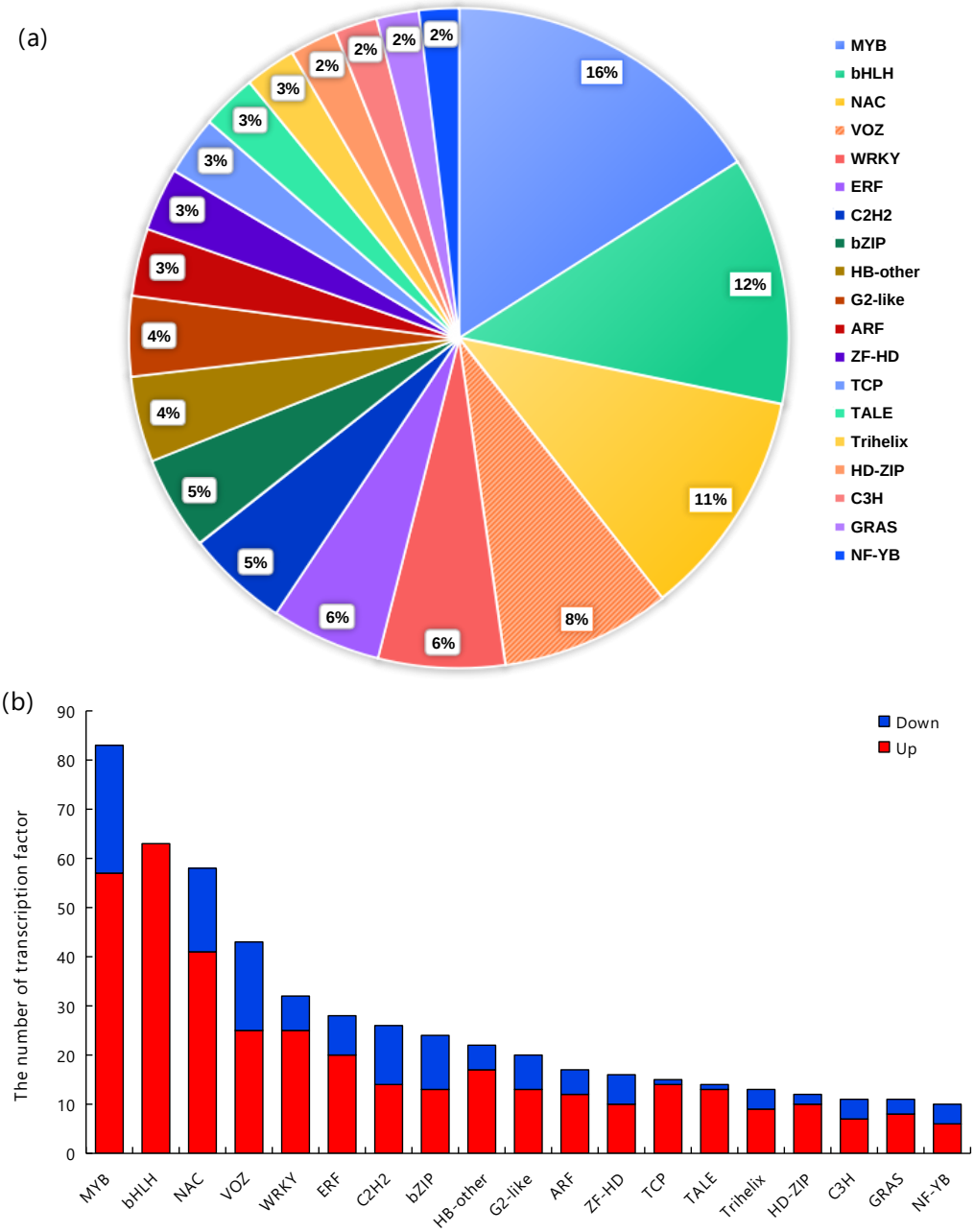

Figure 4. *The classification of differentially expressed transcription-factor genes at the 28 day after pollination. (a) Classification of transcription-factor genes between CK and E; (b) Differentially expressed transcription-factor genes between CK and E, red dots represent up regulation and blue dots shows down regulations*

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Real-time fluorescence quantitative PCR (qRT-PCR) was performed to verify the expression levels of DEGs from the RNA-seq data. The results showed that the anthocyanin content and expression levels of anthocyanin biosynthesis-related genes in purple Se-enriched waxy corn were higher than those in common purple waxy corn (*Figs. 1* and *3a*). In this study, 12 DEGs were selected from phenylpropanoid biosynthesis (zma00940) and flavonoid biosynthesis (zma00941) pathways for qRT-PCR verification, including 10 structural genes, *PAL* (*LOC100273579*), *C4H* (*LOC100272801*), *CHS* (*LOC100282642*), *F3H* (*LOC542712*), *DFR* (*LOC100272982*), *ANR* (*LOC100280098*), *ANS* (*LOC100127010*), *4CL* (*LOC542166*), *F5H* (*LOC103643375*), and *COMT* (*LOC100125646*) and 2 potential transcription factor genes, namely *MYB* (*LOC103636410*) and *bHLH* (*LOC542563*). The expression levels of these 12 DEGs (eight upregulated and four downregulated genes) were detected by qRT-PCR (*Fig. 5*), and the results showed that the expression levels of these 12 genes were consistent with those in the RNA-seq data (*Fig. 3a*), thus supporting the reliability of the data.

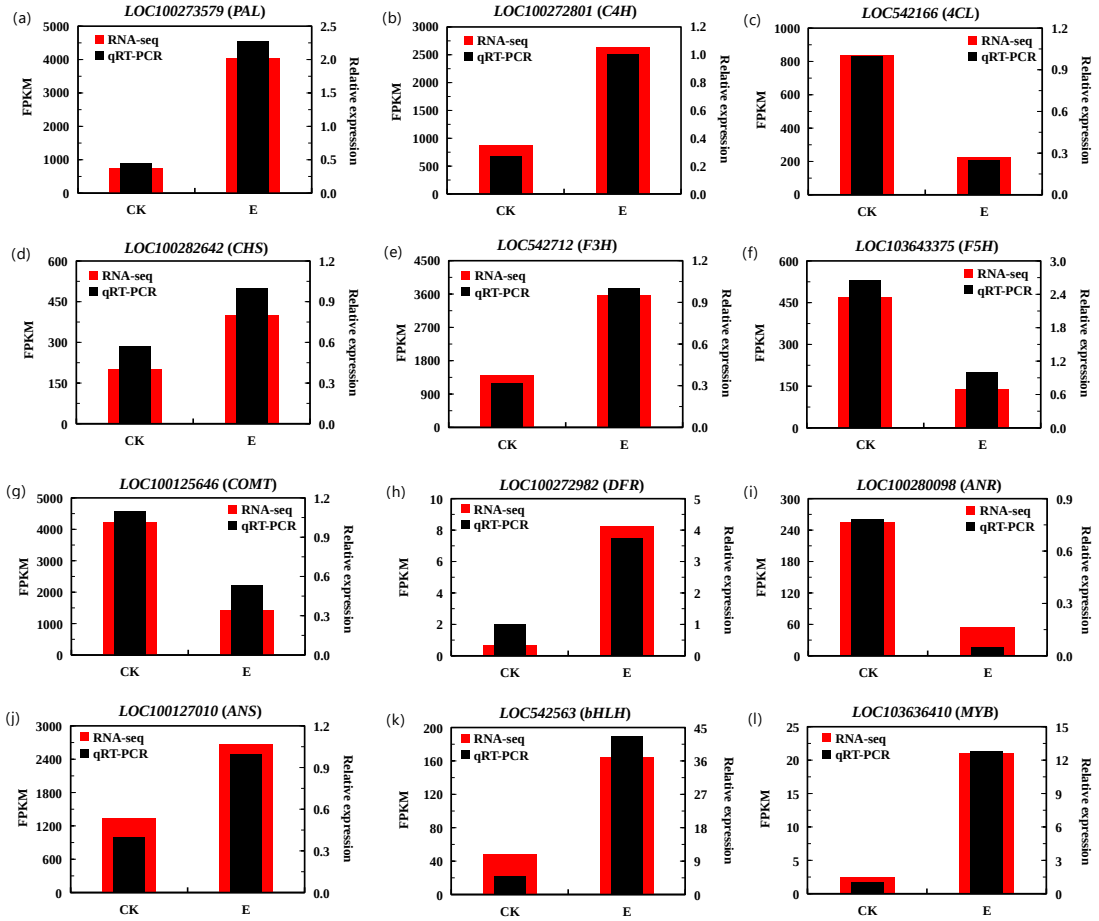

Figure 5. *Quantitative real-time PCR verification at 28 d after pollination*

The expression of the anthocyanin synthesis genes *PAL*, *C4H*, *CHS*, *F3H*, *DFR*, *ANS*, *bHLH*, and *MYB* was significantly higher in purple Se-enriched waxy corn than in common purple waxy corn. One of the branches of anthocyanin synthesis, the lignin

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pathway synthesis genes, namely *4CL*, *F5H*, and *COMT* showed higher expression levels in common purple waxy corn. The other branch, the proanthocyanidin synthesis gene *ANR*, also showed higher expression levels in purple waxy corn. Dou et al. (2021) reported that high Se concentrations inhibit the expression of genes associated with lignin biosynthesis in maize seedlings, thereby negatively regulating lignin deposition. Furthermore, Se treatment inhibited the expression of a key gene (COMT) required for lignin biosynthesis in Alfalfa (*Medicago sativa* L.) (Zhang et al., 2023). These studies elucidate the negative effects of Se on lignin deposition at the gene level.

Conclusion

In summary, natural Se-enriched waxy corn (Jinnuo8) contains more anthocyanins, Se, sugar, and starch than common purple wary corn (Jinnuo20). The most abundant anthocyanin compounds identified were the 6-O-malonyl-beta-D-glu and 3-O-glu forms of Cy, Pn, and Pg. Moreover, the higher expression of *PAL*, *C4H*, *CHS*, *F3H*, *DFR*, *ANS*, *bHLH*, and *MYB*, and lower expression of *4CL*, *F5H*, and *COMT* were largely responsible for the high levels of the 6-O-malonyl-beta-D-glu and 3-O-glu forms. Based on the quality index, the optimum eating date was 28 days after pollination. Although the content of anthocyanins and Se in this period was not the highest, it was still higher than that of artificially Se-enriched purple waxy corn.

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