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Abscisic acid and ethylene biosynthesis-related genes are associated with anthocyanin accumulation in purple ornamental cabbage, *Brassica oleracea* var. *acephala*

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Draft

Abstract: Purple ornamental cabbage (*Brassica oleracea* var. *acephala*) is a popular decorative plant, cultivated for its colorful leaf rosettes that persist in cool weather. It is characterized by green outer leaves and purple inner leaves, whose purple pigmentation is due to the accumulation of anthocyanin pigments. Phytohormones play important roles in anthocyanin biosynthesis in other species. Here, we identified 14 and 19 candidate genes putatively involved in abscisic acid (ABA) and ethylene (ET) biosynthesis, respectively, in *B. oleracea*. We determined the expression patterns of these candidate genes by reverse-transcription quantitative PCR (RT-qPCR). Among candidate ABA-biosynthesis-related genes, the expressions of *BoNCED2.1*, *BoNCED2.2*, *BoNCED6*, *BoNCED9.1* and *BoAAO3.2* were significantly higher in purple compared to green leaves. Likewise, most of the ET biosynthetic genes (*BoACS6*, *BoACS9.1*, *BoACS11*, *BoACO1.1*, *BoACO1.2*, *BoACO3.1*, *BoACO4* and *BoACO5*) had significantly higher expression in purple compared to green leaves. Among these genes, *BoNCED2.1*, *BoNCED2.2*, *BoACS11* and *BoACO4* showed particularly strong associations with total anthocyanin content of the purple inner leaves. Our results suggest that ABA and ET might promote the intense purple pigmentation of the inner leaves of purple ornamental cabbage.

Key words: abscisic acid, ethylene, anthocyanin, ornamental cabbage.

Introduction

Ornamental cabbage (*Brassica oleracea* var. *acephala*) is an attractive cool-weather decorative plant of the Brassicaceae family. A form of cabbage whose leaves do not develop into a typical head, it is more closely related to wild cabbage than to the domesticated types (Huaiming et al. 1995). Ornamental cabbage is widely used in potted plant or cut form due to its attractive foliage color and ability to grow at low temperatures during cool weather (Wang et al. 2011). In addition, its foliage has a long-lasting ornamental period (3–4 months) (Ren et al. 2015). It is also known as ‘leaf peony’, ‘flowering kale’ and ‘ornamental kale’, and shows a range of foliage colors including red, purple, lavender, white, blue and violet (Wei et al. 2008; Zhang et al. 2012; Ren et al. 2015). Purple ornamental cabbage is characterized by brilliant purple inner leaves surrounded by outer green leaves (Liu et al. 2017; Jin et al. 2018a). The purple pigmentation of the inner leaves is due to an accumulation of anthocyanins (Zhang et al. 2012; Jin et al. 2018b, 2018a). Ornamental cabbage is also edible, although its taste is not as good as that of cabbage traditionally cultivated for human consumption (Bergfeldt 2013; Fetouh 2018).

Anthocyanins are plant-derived bioactive compounds belonging to the flavonoid class of secondary metabolites and are responsible for the red, purple and blue colors of reproductive, as well as vegetative, organs of plants (Middleton et al. 1993; Deluc et al. 2006; Rahim et al. 2014). The differences in colors produced by anthocyanins depends on the pH of the vacuole, co-pigments including flavonols and flavones, and metal ions, as well as cell shape (Mol et al. 1998). In addition to providing brilliant pigmentation, anthocyanins help plants in pollination and seed dispersal, and protect from biotic and abiotic stresses (Pourcel et al. 2007).

Anthocyanin biosynthesis and its transcriptional regulation have been well studied in many plant systems including the Brassicaceae species *Arabidopsis thaliana* and *B. oleracea*

(Baudry et al. 2006; Yuan et al. 2009; Rahim et al. 2014, 2017, 2019; Jin et al. 2018b). Expression of the anthocyanin biosynthetic genes is coordinately regulated by three transcription factor (TFs) families through formation of a MYB-bHLH-WD40 transcriptional complex (Baudry et al. 2006; Allan et al. 2008). In addition to these three TF families, several other TFs were also recently reported to be associated with anthocyanin biosynthesis, including bZIP (HY5, ELONGATED HYPOCOTYL 5), MADS box and WRKY (Gyula et al. 2003; Jaakola 2013; Schluttenhofer and Yuan 2015).

Plants also accumulate anthocyanin pigments in different organs to cope with environmental stresses (Chalker-Scott 1999). Low temperature induces anthocyanin accumulation in maize seedlings, which also correlates with higher expression of the structural genes of the anthocyanin biosynthetic pathway (Christie et al. 1994). Recently, Zhang et al. (2018) reported that low temperature enhanced anthocyanin and proanthocyanidin biosynthesis to cope with oxidative damage in strawberry.

Plant hormones play an important role in anthocyanin biosynthesis (Das et al. 2012; An et al. 2018). For example, abscisic acid (ABA), ethylene (ET), cytokinins (CK), jasmonate (JA) and brassinosteroids (BR) enhance anthocyanin production by activating the structural genes of the anthocyanin biosynthetic pathway (Deikman and Hammer 1995; El-Kereamy et al. 2003; Loreti et al. 2008; Shan et al. 2009; Shen et al. 2014; An et al. 2018), whereas auxin (IAA) and gibberellins (GA) decrease anthocyanin production by suppressing these genes (Loreti et al. 2008; Ji et al. 2015). However, little is known about the role of ABA and ET in anthocyanin biosynthesis in brassica crops, including ornamental cabbage. The aim of this study was to identify genes related to ABA and ET biosynthesis and to determine their expression patterns in the inner purple and outer green leaves of ornamental cabbage.

Materials and methods

Experimental plant materials

The purple and green leaves of an ornamental cabbage line (SCNU-OC-41-102), previously reported as purple ornamental cabbage with purple inner leaves by our research group (Jin et al. 2018a), were used in this study (Fig. 1a). Seeds were obtained from Department of Horticulture, Sunchon National University, Republic of Korea, and sown in a nursery soil mixture in a plant growth chamber. After 28 days, the seedlings were moved to a glasshouse. Purple (inner, third leaf) and green (outer, 11th leaf) leaves of a 67-day-old plants were sampled and immediately frozen in liquid nitrogen, and stored at -80 °C.

Identification of ABA and ET biosynthesis-related genes

Phylogenetic analysis was performed to select the ABA- and ET-biosynthesis-related genes using the maximum-likelihood method with 1000 bootstrap values by MEGA6.0 (Tamura et al. 2013). Since the ornamental cabbage (*Brassica oleracea* var. *acephala*) genome is not publicly available, the *B. oleracea* genome database 'Bolbase' (Yu et al. 2013) was used as a reference. Subsequently, the microsyntenic relationships of ABA and ET biosynthetic genes among *B. oleracea*, *B. rapa* and *A. thaliana* were identified using the BLAST search against the genomes of these species. The chromosomal locations of selected genes were retrieved from the *B. oleracea* Genome Database 'Bolbase' v1.0 and 'Phytozome' *B. rapa* FPsc v1.3 (Goodstein et al. 2011), and the relationships among the three species were plotted with 'Circos' v-0.69-6 software with default parameters (Krzywinski et al. 2009).

Determination of total anthocyanin content

Total anthocyanins were isolated from the purple (inner) and green (outer) leaves of ornamental cabbage with methanol containing 1% HCl at room temperature. The leaves were

ground in liquid nitrogen and transferred into the methanol/HCl solution. The mixtures were then homogenized and centrifuged at 14,000 rpm for 14 min. The absorbance of the supernatant was measured spectrophotometrically at wavelengths of 530 nm and 657 nm. Anthocyanin content was presented as total anthocyanins = $(A_{530} - 0.25 \times A_{657}) \times FW^{-1}$, where A_{530} = absorbance at 530 nm; A_{657} = absorbance at 657 nm; and FW = fresh weight of leaves (g) (Mehrtens 2005).

Total RNA isolation and cDNA synthesis

The leaf tissues were ground into a fine powder with a mortar and pestle in liquid nitrogen. 100 mg of leaf tissue powder was used for isolation of RNA using a commercial kit (RNeasy mini, Qiagen, USA) according to the manufacturer's instruction. RNA quantity and integrity were checked with a Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA) and gel electrophoresis. A total of 1 µg total RNA was used for cDNA synthesis using the SuperScript® III First-Strand Synthesis System (Invitrogen, Gaithersburg, MD).

Gene expression analysis by RT-qPCR

The expression profiling of selected genes was carried out using RT-qPCR with 'LightCycler 96' (Roche, Mannheim, Germany). The qRT-PCR reactions were performed using '2x qPCRBIO SyGreen Blue Mix Lo-ROX' (PCR Biosystems Ltd., London, UK) with gene-specific primers (Table 1) and 45 ng/µl cDNA as templates. The gene-specific primers were designed using the Bolbase (Yu et al. 2013) *B. oleracea* genomics database. The RT-qPCR reaction condition was an initial 95 °C for 5 min for enzymatic activation followed by 50 cycles of 95 °C for 10 s, 60 °C for 10 s and 72 °C for 15 s. The gene expression profiling in this study was performed with three independent biological and three technical replicates.

The Cq values obtained by qRT-PCR were analyzed with the LightCycler 96 program (Roche, Germany). The relative expression of the selected genes was analyzed using the comparative $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001) where *BoActin* was used as an internal control (reference gene).

Statistical analysis

Analysis of variance and tests for significance were performed using Minitab 17 (Minitab Inc., USA). The mean separation was accomplished with the Tukey's pairwise comparison.

Results

High leaf anthocyanin content correlates with purple color

We determined the anthocyanin levels in purple (inner) and green (outer) leaves of ornamental cabbage. The results confirmed that total anthocyanin content was significantly higher in purple leaves compared to green leaves of ornamental cabbage (Fig. 1b).

Phylogenetic analysis reveals 14 ABA and 19 ET biosynthetic gene candidates in *Brassica oleracea*

We performed an intensive phylogenetic analysis to identify candidate genes related to ABA and ET biosynthesis. We selected Arabidopsis ABA- and ET-biosynthesis-related genes according to Baron et al (2012), and Booker and DeLong (2015), respectively. To accomplish this, we performed BLAST analysis of the protein sequences of *Arabidopsis thaliana* retrieved from the Arabidopsis Information Resource (TAIR, <https://www.arabidopsis.org/>) against the *B. oleracea* and *B. rapa* genomes using the *B. oleracea* Genome Database 'Bolbase' v1.0 (Yu et al. 2013), the Plant Genomics Resource 'Phytozome' FPse v1.3

(Goodstein et al. 2011) with default parameters, respectively. We aligned the protein sequences with MUSCLE program and visualized them in a phylogenetic tree using the maximum-likelihood method with 1000 bootstrap values by MEGA6.0 (Tamura et al. 2013). The *B. oleracea* genes were named following the *A. thaliana* nomenclature. We identified a total of 14 candidate genes as potentially involved in ABA biosynthesis in *B. oleracea* on the basis of their homology to known genes of *A. thaliana* (9) and *B. rapa* (15) (Fig. 2). These consisted of two *zeaxanthin epoxidase* (ZEP, also called *ABA1*)-like sequences (*BoABA1.1* and *BoABA1.2*), one *xanthoxin dehydrogenase* (*BoABA2*), one *molybdenum cofactor sulfurase* (*BoABA3*), eight *9-cis-epoxycarotenoid dioxygenases* (*BoNCED2.1*, *BoNCED2.2*, *BoNCED3.1*, *BoNCED3.2*, *BoNCED5*, *BoNCED6*, *BoNCED9.1* and *BoNCED9.2*), and two *abscisic-aldehyde oxidase* (*BoAAO3.1* and *BoAAO3.2*) genes. Likewise, we identified 19 candidate genes for the ET biosynthetic pathway in *B. oleracea* (Fig. 3). Among these, eleven and eight were *1-aminocyclopropane-1-carboxylic-acid synthase* (*BoACS2*, *BoACS5.1*, *BoACS5.2*, *BoACS6*, *BoACS7.1*, *BoACS7.2*, *BoACS8.1*, *BoACS8.2*, *BoACS9.1*, *BoACS9.2* and *BoACS11*) and *aminocyclopropane-1-carboxylate oxidase* (*BoACO1.1*, *BoACO1.2*, *BoACO2.1*, *BoACO2.2*, *BoACO3.1*, *BoACO3.2*, *BoACO4* and *BoACO5*)-like genes, respectively.

ABA and ET biosynthetic genes are closely related among Brassicaceae

Next, we constructed a microsynteny map of the orthologous ABA and ET biosynthesis genes among *B. oleracea*, *B. rapa* and *A. thaliana* (Fig. 4) to understand the evolutionary relationships among them. There were 26 orthologous gene pairs (9 ABA and 12 ET biosynthesis-related) between *B. oleracea* and *A. thaliana*, and 36 pairs (14 ABA and 19 ET biosynthesis) between *B. oleracea* and *B. rapa*. This result revealed that the *B.*

180 *oleracea* genes related to both ABA and ET biosynthesis are closely related to those of *B.*
 181 *rapa* and *A. thaliana*.

182

183 **FPKM analysis shows differences in ABA and ET biosynthetic gene expression between** 184 **green and purple leaves**

185 We performed an initial check of the expression of the selected putative candidate
 186 ABA and ET biosynthesis genes using the fragments per kilobase of transcript per million
 187 mapped reads (FPKM) values of the ornamental cabbage transcriptome data previously
 188 reported by our laboratory (Jin et al. 2018a) (Fig. 5 , 6 and Table S1). Among the ABA
 189 biosynthetic genes, five genes, including *BoNCED2.1* (Bol037062), *BoNCED2.2*
 190 (Bol009433), *BoNCED6* (Bol007451), *BoNCED9.1* (Bol027485) and *BoAAO3.2*
 191 (Bol040907), showed a relatively higher expression in purple leaves compared to green
 192 leaves, while the opposite pattern was found for the remaining nine genes. As regards
 193 ethylene biosynthesis, most of the ethylene biosynthetic genes showed a relatively higher
 194 expression in purple compared to green leaves of both ornamental cabbage lines, with the
 195 exception being *BoACO3.2* (Bol010411). Among the *ACS* genes, however, six were not
 196 expressed in either line: *BoACS2* (Bol040692), *BoACS5.1* (Bol008070), *BoACS5.2*
 197 (Bol005726), *BoACS7.1* (Bol042277), *BoACS7.2* (Bol039610) and *BoACS9.2* (Bol037340).

198

199 **RT-qPCR analysis confirms gene expression differences between green and purple** 200 **leaves**

201 We further selected 15 candidate genes based on RNA-seq expression (FPKM value)
 202 data to determine their expression pattern by qRT-PCR (Fig. 7 and 8). The expression of five
 203 genes related to ABA biosynthesis, *BoNCED2.1* (Bol037062), *BoNCED2.2* (Bol009433),
 204 *BoNCED6* (Bol007451), *BoNCED9.1* (Bol027485) and *BoAAO3.2* (Bol040907), was

significantly higher in inner purple leaves compared to the outer green ones. However, the greatest difference in expression levels between purple outer and green inner leaves was found in *BoNCED2.1* and *BoNCED2.2*. The transcript levels of genes related to ET biosynthesis (*BoACS6*, Bol006780; *BoACS9.1*, Bol016984; *BoACS11*, Bol015102; *BoACO1.1*, Bol045912; *BoACO1.2*, Bol022740; *BoACO3.1*, Bol031342; *BoACO4*, Bol040946; *BoACO5*, Bol019026) were significantly higher in purple (inner) leaves compared to green (outer) leaves of purple ornamental cabbage. Nevertheless, the expression of *BoACS8.2* (Bol032729) and *BoACO2.2* (Bol011006) was not significantly different between purple and green leaves. Among these ET biosynthetic genes, *BoACS11* and *BoACO4* showed the highest expression levels in purple leaves compared with green ones.

Discussion

In this study, we reported candidate genes putatively involved in ABA and ET biosynthesis, their expression patterns in the inner purple and outer green leaves of ornamental cabbage, and the relation of these expression patterns to anthocyanin production.

The quantification of total anthocyanins revealed a significantly higher anthocyanin level in the purple inner (younger) leaves compared to the outer green (older) leaves of ornamental cabbage (Fig. 1b). The result suggests that leaf age might influence the anthocyanin accumulation in ornamental cabbage. Cevahir et al. (2004) reported higher anthocyanin content in the younger leaves compared with older leaves in an ornamental species (*Gazania splendens*). We reported a similar result in a previous study on ornamental cabbage, in which we also found higher expression levels of anthocyanin biosynthetic genes, specifically *PAL2*, *C4H2*, *4CL1*, *CHI2*, *UFGT24* and *GTS21*, in the purple inner (younger) leaves compared to the outer (older) green leaves (Jin et al. 2018a). In another study with a different variety of ornamental cabbage (Jin et al., 2018b), we also observed the highest total anthocyanin

content in red as leaves compared to light red and white ones. Moreover, it has been shown that ABA contents correlate the total anthocyanin levels in the red skin of apple cultivar ‘Campbell Redchief’ (Li et al. 2018).

Recent research reveals that in addition to the biosynthetic and regulatory genes of anthocyanin biosynthesis, plant hormones also have important roles in anthocyanin production (Reviewed in Kim et al. 2006; He et al. 2010; Jaakola 2013). ABA plays a vital role in plant growth and development and in adaptation to biotic and abiotic stress conditions (Reviewed in Lee and Luan 2012; Vishwakarma et al. 2017). The application of exogenous ABA significantly increases anthocyanin levels, while treatment with nordihydroguaiaretic acid (NDGA), an ABA biosynthetic inhibitor, blocks anthocyanin accumulation in red sweet cherry (Shen et al. 2014). Moreover, ABA treatment improves fruit skin color in addition to increasing the anthocyanin and total phenolic content in the fruit skin of litchi (Lai et al. 2014) and grapes (Koyama et al. 2014; Neto et al. 2017). Several previous studies revealed that the R2R3-MYB TF is the main regulator of anthocyanin biosynthesis in several different plant species, including BoMYB2 in red cabbage (Yuan et al. 2009), BoPAP2 in ornamental cabbage (Jin et al, 2008b), Rosea1, Rosea2, and Venosa in snapdragon (Schwinn et al. 2006), MYB10 in apple (Espley et al. 2009), LcMYB1 in litchi (Lai et al. 2014), DcMYB6 in carrot root (Xu et al. 2017). ABA directly regulates the expression of anthocyanin-promoting *MYB* genes at the transcriptional level (Shen et al. 2014; Leng et al. 2014). Shen et al. (2014) found that the transcript level of *PacMYBA* was 15.2-fold higher in ABA-treated (2 h pre-incubation) than that in NDGA-treated sweet cherry fruits. Moreover, endogenous ABA levels promote color development in strawberry at ripening by accelerating ethylene production and increasing activity of *phenylalanine ammonia-lyase* (*PAL*), one of the key enzymes of the anthocyanin biosynthetic pathway (Jiang and Joyce 2003). Our results showed significantly higher transcript levels of ABA biosynthetic genes (*BoNCED2.1*,

BoNCED2.2, *BoNCED9.1* and *BoAAO3.2*) in the inner purple leaves compared to the outer green leaves, suggesting that ABA production is higher in purple leaves (Fig. 7). This suggests that the ABA biosynthetic genes might influence the intense purple pigmentation in the inner leaves of the purple ornamental cabbage. The higher expression of *NCED* genes is significantly correlated with higher levels of ABA (Soar et al. 2004; Cramer 2010). Furthermore, Shen et al. (2014) have shown that the expression of the structural as well as the regulatory (*PacMYBA*) genes of anthocyanin biosynthesis, as well as *PacNCED1*, which encodes an enzyme involved in the first step of ABA biosynthesis, was down-regulated in the RNAi fruit sections compared to the control in sweet cherry fruit. Ornamental cabbage is usually grown during winter, and ABA is known to be involved in adaptation to environmental stresses, including cold (Baron et al. 2012; Vishwakarma et al. 2017). It is well-known that cold stress can mildly increase the endogenous ABA level in the plants (Miura and Furumoto 2013). Therefore, it is possible that the younger inner leaves might have increased ABA biosynthesis under cold stress, resulting in the deep purple coloration of these inner leaves. Anthocyanin levels are promoted by low temperature (Leyva et al. 1995), and anthocyanins are reported to assist plants in adapting to and resisting environmental stresses, such as exposure to UV light, cold, drought, pests and diseases (Bi et al. 2018). Abiotic-stress-induced anthocyanins are known to act as reactive oxygen species (ROS) inhibitors and photoprotectants (Kovinich et al. 2015).

The plant hormone ET also promotes anthocyanin production. El-Kereamy et al. (2003) have shown that the exogenous application of an ET-releasing compound (such as 2-chloroethylphosphonic acid, 2-CEPA) on grape berries (cv. ‘Cabernet Sauvignon’) at veraison (the onset of ripening) enhances the anthocyanin content and skin color, while application of 1-methylcyclopropene (1-MCP) decreased ethylene production via blocking of the ethylene receptors. They also found that ET can activate the transcription of the structural

280 genes in the anthocyanin biosynthetic pathway (El-Kereamy et al. 2003). In this study, we
281 found a relatively higher expression of ET biosynthetic genes in inner purple (younger)
282 leaves compared to outer green (older) ones (Fig. 8). The higher transcript levels of ET
283 biosynthetic genes might be due to a cold response. It has been demonstrated that low
284 temperature (2 °C) treatment enhanced ET levels in carnation (Barrowclough et al. 1991).
285 Furthermore, the expression of ET biosynthetic genes might vary along with leaf
286 developmental stages. Rodrigues-Pousada et al. (1993) also reported that the expression of
287 *ACSI* was higher in younger tissues including young leaves in *Arabidopsis*.

288 Both ABA and ET biosynthesis were regulated by the environmental stresses,
289 including cold (Berens et al. 2019; Shinshi 2008). Plants show differential stress responses,
290 depending on the age and developmental stages (Berens et al. 2019). In *Arabidopsis*, it has
291 been shown that younger leaves had higher levels of salicylic acid and immunity to stress
292 compared to older leaves (Berens et al. 2019). The ethylene responsive factor (ERF), an ET
293 inducible TF that regulates the transcription of stress responsive as well as ABA
294 biosynthesis-related genes via interacting with various cis-elements and promotes plant
295 responses to stress (Chen and Zhu 2004; Wu et al. 2007). In *Arabidopsis*, three *CBF* genes
296 belonging to the ethylene-responsive element binding factor/AP2-type TF family are induced
297 within a short period of exposure to cold stress (Gilmour et al. 1999) and stimulate
298 transcription of cold-regulated genes (COR) via binding the promoter region (Zhao et al.
299 2009; Stockinger et al. 1997). The ET and JA signaling pathways can regulate the *CBF* genes
300 (reviewed in Shi et al. 2015). Anyway, although ET has been associated in cold stress
301 response, the precise role of this hormone in cold tolerance m remains largely unknown (Shi
302 et al. 2012). ET signaling seems to negatively regulate the cold tolerance in *Arabidopsis* (Shi
303 et al. 2012) while in tomato, transgenic plants overexpressing *LeERF2* exhibited an enhanced
304 cold tolerance (Zhang and Huang 2010). Moreover, ET can induce the anthocyanin

accumulation in apple skin during fruit ripening (Faragher and Brohier 1984; An et al. 2018). A crosstalk between ABA and ET is also known to operate in a synergic manner in the production of anthocyanin (Wang et al. 2007).

The central core of the ornamental cabbage turn purple in response to the low temperature the plant is sensing. Probably, the cold stress triggers the up-regulation of a various array of defense responses acted by the plant in order to cope with the low temperatures, stimulating the acquisition of a cold tolerance phenotype. These defenses might belong to the ET and ABA domains. The reason why only the inner leaves are purplish is due to their intrinsic vulnerability correlated to their young age. The young inner leaves arranged in the central rosette are more prone to develop chilling injuries, therefore they might accumulate secondary metabolites including anthocyanin with a protective effect in order to scavenge the ROS generated by the increased permeability of the chloroplast membrane. Moreover, the chloroplasts present in the tissues that are experiencing the cold stress are also more vulnerable to the light stress as they already weakened by the cold stress itself, so in order to avoid an excess of visible light the production of a photoprotective anthocyanic screen seems to be an affordable solution to the problem. The purple color is disappear as leaves become mature (outer leaves), and more resistant because of a thicker cutin, higher concentration of soluble solid and development of cold tolerant traits. Mature leaves will turn green, becoming photosynthetically active and sustaining the plant growth.

Conclusions

We identified 14 and 19 putative candidate genes involved in ABA and ET biosynthesis, respectively, in the purple ornamental cabbage *Brassica oleracea* var. *acephala* via an intensive phylogenetic analysis. RNA-seq (FPKM value) and RT-qPCR analysis of the expression patterns of these genes indicated that the ABA biosynthetic genes *BoNCED2.1*

and *BoNCED2.2* and the ET biosynthetic genes *BoACS11* and *BoACO4* were strongly expressed in the purple inner younger leaves compared to the green outer older leaves. These findings correlate the expression of these plant hormone biosynthetic genes with the intense purple pigmentation in the younger inner leaf of purple ornamental cabbage. Therefore, the cross-link between ABA-ET signaling components might provide a possible clue for differential anthocyanin biosynthesis in purple inner and outer green leaves of the same ornamental cabbage.

Conflict of interest

The authors declare no conflict of interest.

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Author contribution

ISN, JGK, JIP, HTK and MAR designed the experiments. JSW conducted the experiments. HJJ and KSA contributed to the experiments. MAR performed the bioinformatics analysis. JSW and MAR analyzed the experimental data. MAR wrote the manuscript. All authors read and approved the manuscript.

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Draft

Table 1. List of primers used in the qRT-PCR analysis.

Sl. no.	Gene name	Forward primers (5'-3')	Reverse primers (5'-3')	Gene accession	Amplicon length (bp)
1	<i>BoNCED2.1</i>	TTGGGGATAATGCCGAAAGACG	ATAGAATCCGCCGGCGACATACAC	Bol037062	155
2	<i>BoNCED2.2</i>	CGTAACGGCGCAAATCCAATG	GAAAACCGGTCTACCCAATCGTGT	Bol009433	168
3	<i>BoNCED6</i>	AACCCGCCCCGATACGATTTTCAG	CCTTCCGGTTCGTTTCTTTTGTC	Bol007451	100
4	<i>BoNCED9.1</i>	CACGTGGGCTTTTCGGTTTACTCG	TCTCCGGTTTGGGTGACTTTGACG	Bol027485	139
5	<i>BoAAO3.2</i>	GTCCGAGCAGAACGTCCTTGAG	CCAGCAGCTTCCGTGTAAACTTG	Bol040907	169
6	<i>BoACS6</i>	GAGTTTATCAGCGTCGCAGAAGTA	CCACCCGGTCATTGTAAGAGTA	Bol006780	142
7	<i>BoACS8.2</i>	ACAACAACCCGAACCGAACTAAAC	TGAAACCAGGAGAAGCGAAAACAG	Bol032729	112
8	<i>BoACS9.1</i>	TGGCGATGCAAAGGCTCAAAGAA	TCATCAGGTACACGGTCGGTCCAT	Bol016984	151
9	<i>BoACS11</i>	TCAGCGGCGACCAAAATGT	AACCCGAAACCAACCTGTCTCTT	Bol015102	154
10	<i>BoACO1.1</i>	GGTTCCTATCCCGCCATCA	ACAGCACTTTTGTATCTCCCATTA	Bol045912	93
11	<i>BoACO1.2</i>	ATGAGGAGCATTTGAAAGAGAAGT	TTATGCCAAACGAAGAAAGTGC	Bol022740	109
12	<i>BoACO2.2</i>	CCAACGGCAGGTACAAGAGTG	TTTACAGGCAAGCGATGAAGC	Bol011006	131
13	<i>BoACO3.1</i>	CAACGGGAAGTACAAGAGCATAAT	CGCATCACTTCCAGGGTTGTA	Bol031342	97
14	<i>BoACO4</i>	GGCGGCATCATCCTCCTCTTC	TCCACGCTCTTGTACTTCCCATTG	Bol040946	158
15	<i>BoACO5</i>	CGATGTTTACGCCGACGCCTAATG	TTTCCTTCCTCCCTCGCCAACAC	Bol019026	117
16	<i>BoActin*</i>	CTGAGGAGCACCCGGTTCTTCTTA	CGGAGGATGGCGTGTGGTAGA	Bol003004	238

*, *BoActin* primer sequences were previously reported by Rahim et al. (2017).

List of Figures

Fig. 1. Phenotype of purple ornamental cabbage line (SCNU-OC-41-102) used in this study

(a). The circles indicate the sampling areas for RNA extraction and pigment quantification.

Total anthocyanin contents in inner purple and outer green leaves (b).

Fig. 2. A phylogenetic tree showing the putative abscisic acid (ABA) biosynthetic genes of *Brassica oleracea* and their homologs in *Arabidopsis thaliana* and *B. rapa*. The tree was built by the maximum-likelihood method using 1000 bootstrap values with MEGA6.0 software (Tamura et al. 2013). The deduced amino acid sequences of these plant species were obtained from ‘Bolbase’ ‘Phytozome’ and ‘TAIR’. The green, red and grey circles denote ABA-biosynthetic genes of *A. thaliana*, *B. oleracea* and *B. rapa*, respectively. The accessions are shown in parentheses for each gene.

Fig. 3. Phylogenetic analysis of putative ethylene biosynthetic genes in *B. oleracea* and their homologs from *A. thaliana*, *B. oleracea* and *B. rapa*. The trees were constructed by the neighbor-joining method using 1000 bootstrap values with MEGA6.0 software (Tamura et al. 2013). The deduced amino acid sequences of these plant species were obtained from ‘Bolbase’ ‘Phytozome’ and ‘TAIR’. The green, red and grey circles indicate ethylene biosynthetic genes in *A. thaliana*, *B. oleracea* and *B. rapa*, respectively. The accessions are shown in parenthesis for each gene.

Fig. 4. Microsynteny analysis of abscisic acid (ABA)- and ethylene (ET)-biosynthesis-related genes among *B. oleracea*, *B. rapa* and *A. thaliana*. The orange, green and blue colors denote *B. oleracea*, *B. rapa* and *A. thaliana* chromosomes, respectively.

Fig. 5. Heatmap illustration of the expression pattern of abscisic acid (ABA) biosynthetic genes in purple and green ornamental cabbage lines based on the FPKM data obtained through RNA sequencing of purple (inner) and green (outer) leaves of ornamental cabbage. The red and blue boxes designate the highest and the lowest expression values, respectively.

Fig. 6. Heatmap illustration of the expression pattern of ethylene biosynthetic genes in purple and green ornamental cabbage lines based on the FPKM data obtained through RNA sequencing of purple (inner) and green (outer) leaves of ornamental cabbage. The red, blue and white boxes designate the highest values, the lowest values, and no expression, respectively.

Fig. 7. Expression profiles of abscisic acid (ABA) biosynthetic genes by qRT-PCR in purple (inner) and green (outer) leaves of ornamental cabbage. Error bars represent $\pm$ SE of the means of triplicates. Letters above the bars indicate significant differences.

Fig. 8. Expression profiles of ethylene biosynthetic genes by qRT-PCR in purple (inner) and green (outer) leaves of ornamental cabbage. Error bars represent $\pm$ SE of the means of triplicates. Letters above the bars indicate significant differences.

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Fig. 4. Microsynteny analysis of abscisic acid (ABA)- and ethylene (ET)-biosynthesis-related genes among *B. oleracea*, *B. rapa* and *A. thaliana*. The orange, green and blue colors denote *B. oleracea*, *B. rapa* and *A. thaliana* chromosomes, respectively.

TranAcc	GeneName	Description	Purple	Green
Bol027222	BoABA1.1	Zeaxanthin epoxidase		
Bol019241	BoABA1.2	Zeaxanthin epoxidase		
Bol037062	BoNCED2.1	9-cis-epoxycarotenoid dioxygenase NCED2		
Bol009433	BoNCED2.2	9-cis-epoxycarotenoid dioxygenase NCED2		
Bol005093	BoNCED3.1	9-cis-epoxycarotenoid dioxygenase NCED3		
Bol035582	BoNCED3.2	9-cis-epoxycarotenoid dioxygenase NCED3		
Bol022516	BoNCED5	Probable 9-cis-epoxycarotenoid dioxygenase NCED5		
Bol007451	BoNCED6	9-cis-epoxycarotenoid dioxygenase NCED6		
Bol027485	BoNCED9.1	9-cis-epoxycarotenoid dioxygenase NCED9		
Bol018961	BoNCED9.2	9-cis-epoxycarotenoid dioxygenase NCED9		
Bol035060	BoABA2	Xanthoxin dehydrogenase		
Bol026459	BoAAO3.1	Absciscic-aldehyde oxidase		
Bol040907	BoAAO3.2	Benzaldehyde dehydrogenase (NAD(+))		
Bol038236	BoABA3	Molybdenum cofactor sulfurase		

Fig. 5. Heatmap illustration of the expression pattern of abscisic acid (ABA) biosynthetic genes in purple and green ornamental cabbage lines based on the FPKM data obtained through RNA sequencing of purple (inner) and green (outer) leaves of ornamental cabbage. The red and blue boxes designate the highest and the lowest expression values, respectively.

TranAcc	GeneName	Description	Purple	Green
Bol040692	<i>BoACS2</i>	1-aminocyclopropane-1-carboxylate synthase 2		
Bol008070	<i>BoACS5.1</i>	1-aminocyclopropane-1-carboxylate synthase 5		
Bol005726	<i>BoACS5.2</i>	1-aminocyclopropane-1-carboxylate synthase 5		
Bol006780	<i>BoACS6</i>	1-aminocyclopropane-1-carboxylate synthase 6		
Bol042277	<i>BoACS7.1</i>	1-aminocyclopropane-1-carboxylate synthase 7		
Bol039610	<i>BoACS7.2</i>	1-aminocyclopropane-1-carboxylate synthase 7		
Bol028890	<i>BoACS8.1</i>	1-aminocyclopropane-1-carboxylate synthase 8		
Bol032729	<i>BoACS8.2</i>	1-aminocyclopropane-1-carboxylate synthase 8		
Bol016984	<i>BoACS9.1</i>	1-aminocyclopropane-1-carboxylate synthase 9		
Bol037340	<i>BoACS9.2</i>	1-aminocyclopropane-1-carboxylate synthase 9		
Bol015102	<i>BoACS11</i>	1-aminocyclopropane-1-carboxylate synthase 11		
Bol045912	<i>BoACO1.1</i>	1-aminocyclopropane-1-carboxylate oxidase 1		
Bol022740	<i>BoACO1.2</i>	1-aminocyclopropane-1-carboxylate oxidase 1		
Bol006636	<i>BoACO2.1</i>	1-aminocyclopropane-1-carboxylate oxidase		
Bol011006	<i>BoACO2.2</i>	1-aminocyclopropane-1-carboxylate oxidase		
Bol031342	<i>BoACO3.1</i>	1-aminocyclopropane-1-carboxylate oxidase 3		
Bol010411	<i>BoACO3.2</i>	1-aminocyclopropane-1-carboxylate oxidase 3		
Bol040946	<i>BoACO4</i>	1-aminocyclopropane-1-carboxylate oxidase 4		
Bol019026	<i>BoACO5</i>	1-aminocyclopropane-1-carboxylate oxidase 5		

Fig. 6. Heatmap illustration of the expression pattern of ethylene biosynthetic genes in purple and green ornamental cabbage lines based on the FPKM data obtained through RNA sequencing of purple (inner) and green (outer) leaves of ornamental cabbage. The red, blue and white boxes designate the highest values, the lowest values, and no expression, respectively.

Fig. 7. Expression profiles of abscisic acid (ABA) biosynthetic genes by qRT-PCR in purple (inner) and green (outer) leaves of ornamental cabbage. Error bars represent $\pm$ SE of the means of triplicates. Letters above the bars indicate significant differences.

136x364mm (300 x 300 DPI)

Fig. 8. Expression profiles of ethylene biosynthetic genes by qRT-PCR in purple (inner) and green (outer) leaves of ornamental cabbage. Error bars represent $\pm$ SE of the means of triplicates. Letters above the bars indicate significant differences.

284x370mm (300 x 300 DPI)