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Red anthocyanins contents and the relationships with phenylalanine ammonia lyase (PAL) activity, soluble sugar and chlorophyll contents in carmine radish (*Raphanus sativus* L.)

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Abstract: Red anthocyanins from Carmine radish is rich both in root flesh and peel and it is relatively simple and efficient to extract these compounds. The accumulation, distribution and content of anthocyanins in root are related to phenylalanine ammonia lyase (PAL) activity, soluble sugar and chlorophyll contents. The results showed that anthocyanins were synthesized at the first day of seed germination and were most abundant in the top of hypocotyls. The content of anthocyanins was higher in the root peel than in flesh and root apex, and in aboveground parts compared with underground sections. The anthocyanins contents in cotyledon grown under light and dark and hypocotyls grown in the dark increased initially and then reduced, and in roots grown under light was higher than in those grown in the dark. Chlorophyll content in leaves fluctuated but increased overall, whereas it was almost unchanged in the petioles. The correlations between anthocyanins content and PAL activity, soluble sugar and chlorophyll contents in different treatments showed positive by Day 4 then negative. These results are helpful to understand the mechanism of anthocyanins biosynthesis in carmine radish.

Keywords: carmine radish; anthocyanins; PAL; soluble sugar; relationship

Anthocyanins are the most important water-soluble natural pigments in different plant species and are responsible for the red, purple, blue and orange colours presented in leaves, flowers, fruits, and other plant parts (Moreno et al. 2005; Schaberg et al. 2017). Anthocyanins are playing relevant roles in plant propagation and ecophysiology. They help to attract pollinators and seed-dispersing animals and assist in plant defending against biological and abiotic stresses (Santos-Buelga et al. 2014), such as bacterial and insect attacks (Edwards et al. 2008), wounding (Jeannette et al. 2000; Gould et al. 2002), drought (Zhang et al. 2007), and nutrient deficiencies (Schaberg et al. 2003). Anthocyanins are also important antioxidant molecules (Gould et al. 2002) and help to protect plants from damage by active oxygen species (Nagata et al. 2003; Teng et al. 2005). They are deemed natural food additives in the industries of foods, beverages and cosmetics, because they not only have many benefits for plants, but also on human health and well-being due to their antioxidant properties and free radical scavenging

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capacity, which can prevent people from coronary heart disease, cardiovascular disease, and cancer (Nemie-Feyissa et al. 2015; Liu et al. 2017).

Natural anthocyanins have been extracted from grape (Nile et al. 2015), raspberry (Pantelidis et al. 2007), red cabbage (McDougall et al. 2007) and red radish (Tatsuzawa et al. 2008). Anthocyanins are thought to be potential replacements for synthetic pigments because of their attractive bright colours, good colour stability and water solubility that allows their utilization in industries (Patil et al. 2009). Radish is an easy vegetable to grow and can be harvested within 4–6 weeks (Giusti et al. 1998a), and the yield of roots is very high. The anthocyanins responsible for red and purple radishes have been successfully extracted and characterized by different researchers (Ishikura, Hayashi 1962; Harborne 1963; Fuleki 1969; Otsuki 2002). The use of red radish anthocyanins extract was assessed (Giusti, Wrolstad 1996) and the colour characteristics were found very stable storage at room temperature (Giusti et al. 1998b).

However, what the previous researches used to extract radish anthocyanins are mainly root peel because of high anthocyanins content. Carmine radish is a local featured cultivar available in Sichuan province, China, and its significant characteristic is rich in red anthocyanins both in peel and flesh. Usually, they are processed by the locals to extract red pigments from the whole radish with very simple methods. Previous studies have focused on the extraction, purification, and the physical and chemical properties of carmine radish pigment (Liu, Zeng 1999; Lv et al. 2001; Wu et al. 2001). However, there have been fewer reports on the physiological and biochemical study of anthocyanins during growth of the carmine radish. Light is an important external environmental factor that affects anthocyanins biosynthesis and content in radish (Mol et al. 1996). The objectives of the present work are to characterize the pigments accumulation process and relationships of anthocyanins with PAL activity, soluble and chlorophyll contents under periods of light and dark during the early seed germination stage, and to identify changes in anthocyanins content in different parts of carmine radish. These results will provide useful information for production of higher anthocyanins content carmine radish, and for further research on the mechanism of regulation and molecular biology of anthocyanins biosynthesis.

MATERIAL AND METHODS

Plant material and treatments. Seeds from carmine radish cultivar 'Yanzhi 3' were obtained from plants growing in Sichuan Province. Field and laboratory experiments were designed to evaluate the effects of light and dark on the synthesis of anthocyanins and PAL activity, and soluble sugar and chlorophyll contents. This experiment was carried out at the College of Agriculture and Biotechnology, Zhejiang University, Hangzhou during 2010. One hundred healthy carmine radish seeds were sown uniformly over a piece of wet filter paper placed in the bottom of a plastic box (L: 17 cm, W: 11 cm, H: 7 cm) and six boxes were used in total, and were divided into two groups (GP). In GP-1, three boxes were kept under a controlled light and dark regimen of 14/10 h at 25 ± 1°C for 15 days; in GP-2, the other three boxes were kept in the dark at 25 ± 1°C for the same length of time. The seeds in each group then germinated under controlled conditions and the seedlings were watered daily with distilled water. Twenty seedlings were selected on the first day of germination and their cotyledons and hypocotyls were dissected and maintained separately every 2 days for 14 days.

Another 150 healthy seeds were sown under field conditions for determining the effect of day and night on the growth and development of carmine radish. The experiment was carried out following a completely randomized design with a distance between rows and plants was 15 cm × 15 cm and the plant area was 2 m^2. The soil used was sandy soil and a mixed fertilizer of N (nitrogen), P (phosphorus), and K (potassium) was applied at a rate of 200 g. Ten uniform and vigorously growing seedlings were first collected on the 7^{th} day of germination and were then collected at weekly intervals. The petioles, leaves, and roots of seedlings were dissected and stored separately. The peel, flesh, petioles, and root apex of each radish were harvested once they had matured. The collected samples were weighed and maintained at –70°C until use. The leaves, petioles, and taproots were dissected to determine the presence of anthocyanins during radish development.

Determination of anthocyanins content. Total anthocyanins content of each tissue sample was determined using the pH-differential method described by Giusti and Wrolstad (2001). A ThermoSpectronic Helios spectrophotometer (ThermoSpectronic, Cambridge, UK) and 1-cm path-length disposable cells were used for spectral measurements at 510 and

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700 nm. Pigment content was calculated as Pg-3-glu, using an extinction coefficient of 31,600 l/cm·mg and a molecular weight of 433.2 g/l (Wrolstad 1976).

Measurement of soluble sugar content. Soluble sugars were determined based on the phenol-sulfuric acid method (Li 2000): 0.2 g fresh weight (FW) of samples was homogenized with 5–10 ml distilled water, and boiled twice for 30 min. The extraction was filtered and a constant volume of 25 ml was obtained. A 0.5-ml extraction was treated with 1 ml 9% phenol and 5 ml 98% sulfuric acid, left to stand for 30 min and then the absorbance at 485 nm was determined using a spectrophotometer (Biochrom 210; Biochrom, China). The soluble sugar content was expressed as mg/g FW.

Measurement of PAL activity. For PAL activity, 1.0 g samples were ground in 7.5 ml 0.2 M borate buffer (pH 8.8, containing 10% PVP, 5 mM β-mercaptoethanol, 1 mM EDTA and 1 mM DTT) on ice (Koukol, Conn 1961). The mixture was centrifuged for 20 min at 12,000 *g* and 4°C, and the supernatant was collected for the enzyme assay. 0.8 ml supernatant was added to 3 ml of reactive solution (pH 8.0) containing 2 ml 0.2 M borate buffer (pH 8.8) and 1 ml 0.05 M L-phenylanine. The mixture was incubated for 90 min at 37°C and then 0.2 ml 6 M hydrochloric acid was added to terminate the reaction. The mixture was centrifuged and the supernatant was used to determine PAL activity at 290 nm. Any increase in OD 290 nm because of the formation of *trans*-cinnamate was measured spectrophotometrically. PAL activity was expressed as a change in unit/h FW, where 1 unit represented a 0.01 increment of A_{290} per minute.

Chlorophyll determination. Chlorophyll content was determined according to the method of Fan et al. (2013). 0.2-g samples were weighed once the leaves or petioles had been cut into pieces and then put into lidded tubes. 10–15 ml 80% acetone was added and then extracted in the dark overnight, followed by 3 to 4 rounds of shaking. The following day, the extraction solution was made up to 20 ml with 80% acetone and then filtered. The mixture was centrifuged and the supernatant was used to determine chlorophyll content at 663 and 645 nm using a spectrophotometer.

Statistical analysis. The experiment followed a completely randomized design with a factorial arrangement with three replications. The experiment was conducted twice; data were pooled, and subjected to statistical analysis using Excel 2007 and DPS 3.01. Each treatment was analysed with at least three repeats and tissue samples represented at least five plants. The least significant difference (LSD) test was applied to compare the treatment means at $P = 0.05$ and 0.01. Graphical presentation of data was carried out using Microsoft Excel. Correlations were determined between anthocyanins levels, PAL activity, soluble sugar content, and chlorophyll content.

RESULTS

Accumulation of, and changes in, anthocyanins content in carmine radish

Fig. 1a shows the accumulation process of anthocyanins during the first 7 days' germination under light. Anthocyanins had appeared in the cotyledon and hypocotyls by the first day of germination and then slowly accumulated in the root via the hypocotyl. The whole hypocotyl had turned red by Day 5, whereas the cotyledon had become green, with red along its edge, by Day 7 after germination. The distribution of anthocyanins in the hypocotyl was non-uniform, with a higher concentration in the top of the hypocotyl compared with the base.

Anthocyanins were distributed in every part of the carmine radish but the quantity varied. Before Day 5 after germination, there was little anthocyanins in the leaves and what was present occurred mainly in the leaf veins (Fig. 1b). The petioles also contained some anthocyanins, mainly in the petiole epidermis (Fig. 1c). By contrast, in the root, there was more anthocyanins in the peel than in the root flesh, with hardly any in the primary xylem (Fig. 1d). During the mature growth stage, anthocyanins occurred mainly in the petiole epidermis (Fig. 2e) but was found throughout the root (Fig 2f, g). However, the distribution of anthocyanins in the root was nonuniform. The anthocyanins content in the root peel was higher than in the root flesh (Fig. 1c, d, f, g), with aboveground sections containing more anthocyanins compared with the underground sections (Fig. 4b).

Analysis of anthocyanins content and other physiological characteristics at the first 7-day germination

PAL activity. PAL activity varied across the different treatments. There were no significant changes in PAL activity in cotyledons grown under either

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Fig. 1. Accumulation of, and changes in, anthocyanin content in seedlings and different parts of carmine radish during plant growth. (a) – seedlings; (b) – leaf vein; (c)– transection of petiole during early growth; (d) – transection of root during early growth; (e) – petiole during late growth; (f) – longitudinal section of root; (g) – transection of underground (left) and aboveground (right) parts of mature radish root (arrow indicates the position anthocyanin accumulates)

light or dark conditions, or during plant growth. In plants grown under the light, PAL activity in the hypocotyl increased initially and then reduced, with the highest activity recorded on Day 4 after germination. By contrast, PAL activity in plants grown in the dark was reduced throughout the plant. In all four treatments, the PAL activity in hypocotyls of plants grown in the dark was significantly lower than in plants grown under the light (Fig. 2a).

PAL activity in leaves initially decreased and then increased to a stable level once growth was fully underway. In roots, PAL activity also increased to a stable level, with PAL activity in the leaves being slightly higher than in the roots. By contrast, PAL activity in the petioles first increased and then reduced, and was lower than in the leaves or roots (Fig. 2b).

Anthocyanins content. Significant differences in anthocyanins content in radish grown under the four different treatments were recorded. The changes of anthocyanins contents in cotyledons grown either under the light or in the dark was similar, in that it first increased before Day 6, 2.3 mg/100 g and 2.2 mg/100 g, and then decreased. By contrast, the anthocyanins content in hypocotyls grown under light was higher than in those grown in the dark, peaking at 2.95 mg/100 g by Day 14 after germination. The anthocyanins content in hypocotyls grown under light was also higher than in cotyledons, whereas that in hypocotyls grown in the dark was generally less (Fig. 2c).

Except for some fluctuations, anthocyanins content in taproots generally increased throughout the growth period, peaking at 22.24 mg/100 g during Week 8 following germination. By contrast, the anthocyanins content in petioles remained stable (Fig. 2d).

Soluble sugar content. There were differences in the soluble sugar contents in cotyledon and hypocotyls grown under light and in dark (Fig. 2e). The soluble sugar content in both cotyledons and hypocotyls grown under light first increased then gradually declined, with levels being higher in hypocotyls than in the cotyledons. By contrast, the soluble sugar content in both cotyledons and hypocotyls grown in the dark decreased, although there were some fluctuations. The soluble sugar content in hypocotyls grown in the dark was lower than in hypocotyls in the other three treatment groups. The soluble sugar content in taproots increased during the growth period, peaking at 26.45 mg/g by Week 12. By contrast, the soluble

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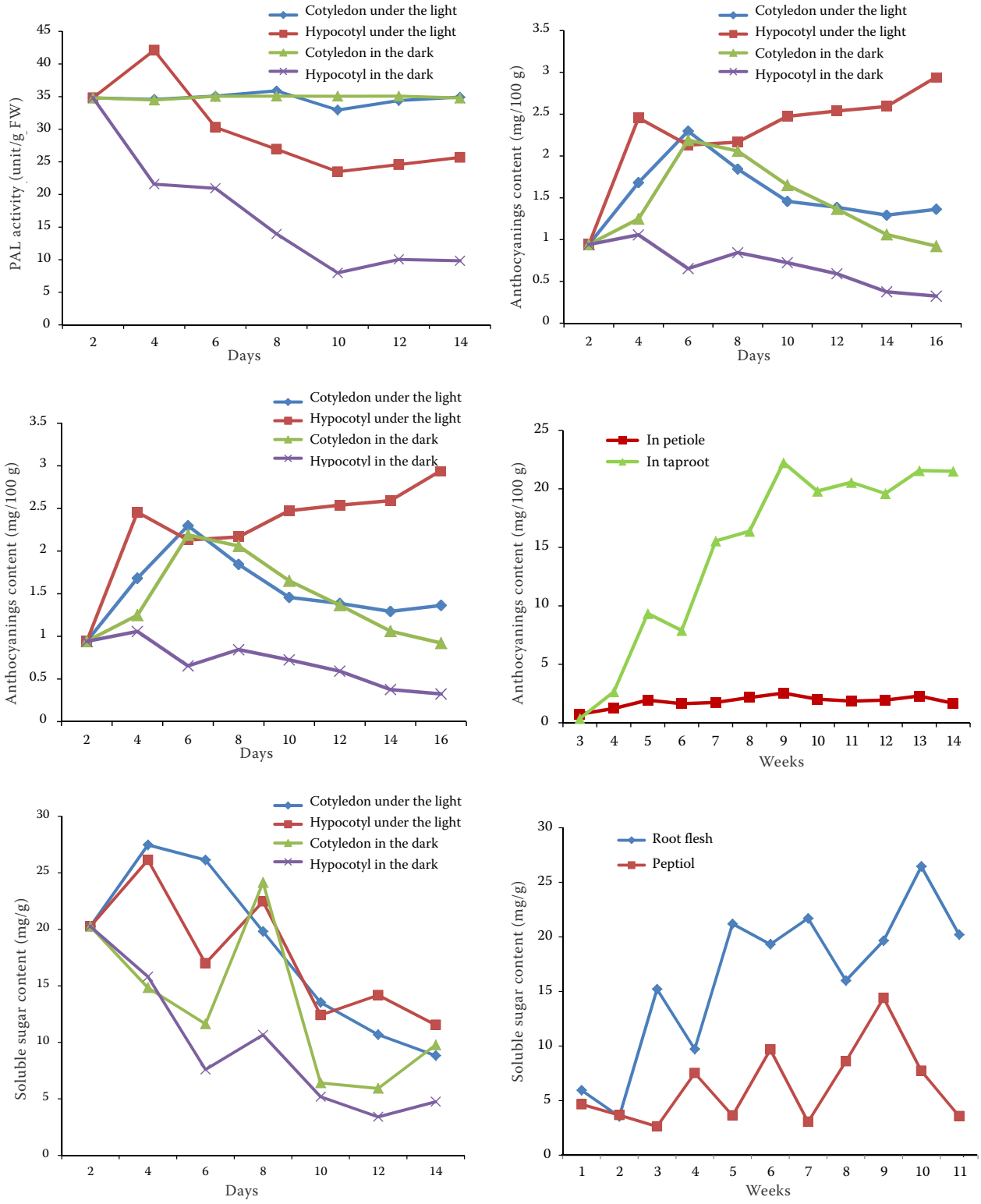

Fig. 2. Changes in PAL activity, and the content of anthocyanins and soluble sugar of carmine radish during growth: (a) changes of PAL activity in four treatments; (b) changes of PAL activity in leaf, petiole and taproot during growth in field; (c) changes of anthocyanin content in four treatments; (d) changes of anthocyanin content in petiole and taproot dring growth in field; (e) changes of soluble sugar content in four treatments; (f) changes of soluble sugar content in petiole an taproot during growth in fieldig

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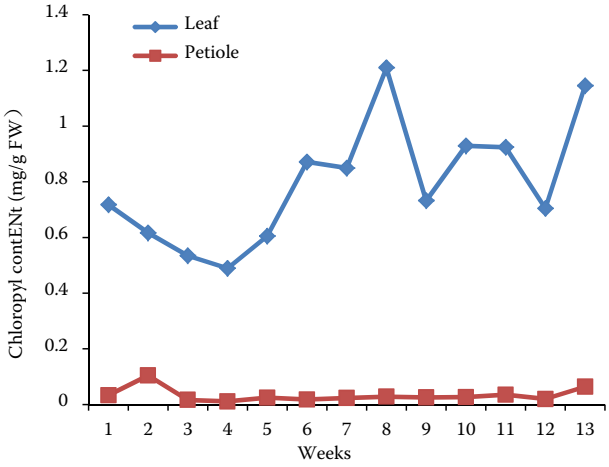

Fig. 3. Changes of the chlorophyll content in leave and petiole of carmine radish during growth in the field. The chlorophyll content in leaf fluctuated a lot but almost unchanged in petiole during growth in the field

sugar content in the petioles fluctuated and was lower than in the roots (Fig. 2f).

Chlorophyll content. The chlorophyll content in leaves and petioles differed significantly in plants grown under field conditions. In the leaves, the chlorophyll content was reduced during the early growth period and then steadily increased, peaking at 1.2 mg/g FW by Week 8. However, no change was observed in the chlorophyll content of petioles, which was generally lower in leaves (Fig. 3).

PAL activity, anthocyanins and soluble sugar contents in different parts of carmine radish. PAL activity was similar in the flesh, peel and leaf of the radish, but significantly higher than in the petiole and root apex. The max. PAL activity (30.69 unit/g FW) was observed in peel, and the lowest in petioles (7.52 unit/g FW) (Fig. 4a). The anthocyanins content in peel was 25.03 mg/100 g, which was higher than in other parts of the radish (Fig. 4b). Significant differences were found in the soluble sugar content among different parts of the radish (Fig. 4c). The soluble sugar content was highest in the flesh (29.44 mg/g), followed by that of peel and leaf (19.23 mg/g and 7.77 mg/g, respectively). The petiole and root apex contained the least amount of soluble sugar. The petiole also contained some anthocyanins, mainly in epidermis, although the levels were low. The colour of the pigment extracted from the petiole was dull-red or purple, and the extraction contained a mix of anthocyanins and green chlorophyll.

Table 1. Correlations between anthocyanin content, PAL activity and soluble sugar content of carmine radish under different treatments

Character	Anthocyanin content (treatment)			
	cotyledon (light)	hypocotyl (light)	cotyledon (dark)	hypocotyl (dark)
Soluble sugar	0.213	–0.311	0.469*	0.874**
PAL activity	0.545*	–0.254	0.452	0.652**

*,**correlation significant at P <0.05 and P <0.01, respectively

Correlation of anthocyanins with PAL activity and soluble sugar content. From the Fig. 2 and Table 1, the results suggested that the relationships between anthocyanins and PAL activity, soluble

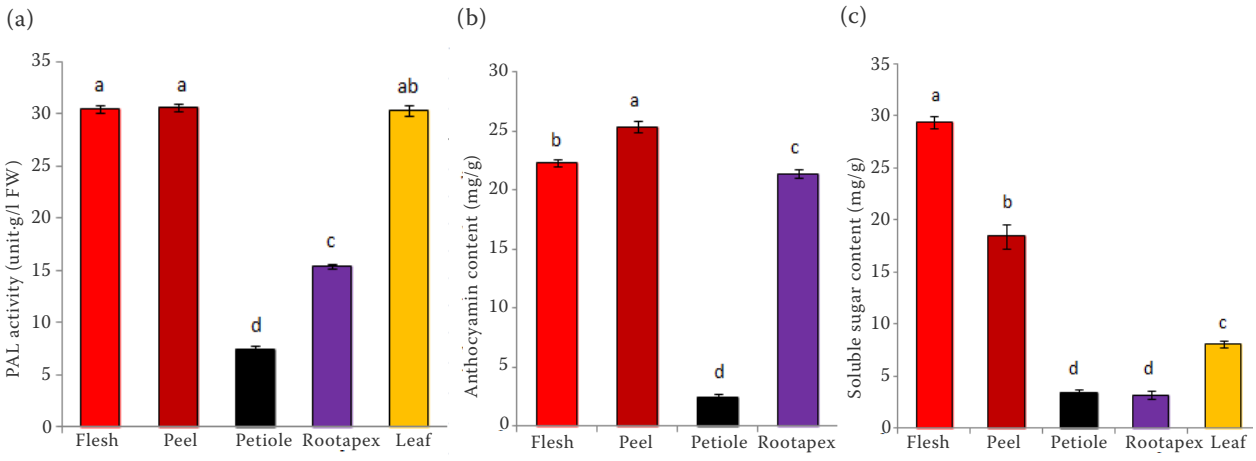

Fig. 4. Differences in PAL activity, and the content of anthocyanins and soluble sugar in different parts of carmine radish: (a) PAL activities in flesh, peel, petiole, rootapex and leaf, (b) anthocyanin contents in flesh, peel, petiole and rootapex, and (c) The soluble sugar content in flesh, peel, petiole, rootapex and leaf

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Table 2. Correlation between anthocyanin content, PAL activity, and soluble sugar and chlorophyll content in the taproot and petiole of carmine radish

Characteristic	Anthocyanin content	
	taproot	petiole
PAL activity	0.609*	0.73*
Soluble sugar content	0.678*	0.241
Chlorophyll content	N/A	0.496

*correlation significant at P <0.05; N/A – not available

sugar were complex in across different treatments. The correlations of anthocyanins with PAL activity were positive by Day 6 and then negative in the treatments of cotyledon treated under the light and dark. In hypocotyls treated in the dark, the anthocyanins content had a significant positive correlation with PAL activity on the whole. In treatments under the light, the relationships of anthocyanins and soluble sugar were all significant positive by Day 4, and then significant positive in hypocotyls, but negative in cotyledons. Treated in the dark, the correlation of anthocyanins with soluble sugar was negative by Day 6 and then negative in cotyledons, but was positive in hypocotyls. Interestingly, the correlation between anthocyanins and PAL activity and soluble sugar content in hypocotyls grown in the dark or under light was different. For plants grown in the dark, there was a significant positive correlation between anthocyanins and PAL activity and soluble sugar content ($P < 0.01$), whereas, this correlation was negative and the differences almost nonsignificant for plants grown under the light.

In taproots, there was a significant positive correlation between anthocyanins and PAL activity and soluble sugar content ($P < 0.05$). This was also true in petioles ($P < 0.05$). In conclusion, there was a positive ($P < 0.05$) correlation between anthocyanins content and PAL activity, and soluble sugar content, although less so in roots and petioles (Table 2).

DISCUSSION

Anthocyanins are the most important group of water-soluble compounds, and they are responsible for multiple colours in different plant organs. In the last few decades, the mechanisms of biosynthesis, regulation and transport of anthocyanins have been identified and functionally characterized in *A.* thaliana (Holton, Cornish 1995). These researches have provided us comprehensive understanding of anthocyanin biosynthesis and revealed the accumulation and metabolic profiles of anthocyanins in the model plant (Winkel-Shirley 2001; Broun 2005; Lepiniec et al. 2006). Carmine radish is an ideal anthocyanin extract raw material due to abundance of red anthocyanins both in peel and flesh, and easy process. Anthocyanins in carmine radish are synthesized from the very beginning of seed germination in the cotyledon and hypocotyls then spread throughout the seedling and accumulate mainly in root peel and flesh as it grows under light. The distribution of anthocyanins in root is nonuniform, being higher in the peel than in the flesh and higher in aboveground than in belowground sections, which is consistent with the results of Meng et al. (2006).

Anthocyanins synthesis in several plant tissues are known to be correlated with closely between the activity of PAL activity and soluble sugar content. The PAL enzyme is the first enzyme in the anthocyanins synthesis pathway, suggesting that one of the ways that light increases anthocyanins production is by stimulating greater PAL activity (Huang et al. 2010). However, the results in this study showed that anthocyanins contents, in cotyledons grown under either light or dark conditions or during plant growth, decreased, but the PAL activity did not change significantly. The apparent discrepancy between anthocyanins contents and PAL activity in this study may have resulted from synthesis of anthocyanins mainly in hypocotyls and treated under the light, taproots instead of in cotyledons and treated in the dark and petioles. The apparent excess of PAL activity in different treatments suggests that, although the enzyme is essential for anthocyanin synthesis, it might not be the rate-limiting enzyme (Cheng 1991). However, PAL is also considered to be a key enzyme controlling the channelling of phenylpropanoid into phenolic synthesis (Malmir 2012), which may result in high PAL activity but low anthocyanin content. The role of sugar is not only a resource for carbohydrate formation, but also acts as signal molecule to activate/repress various reactions, and further affects anthocyanins synthesis (Weiss 2000; Bureau et al. 2009). In addition, the effectiveness of environmental factors, such as light affects signal transduction and the expression of genes involved in anthocyanins biosynthesis (Mol et al. 1996; Hirner

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et al. 2001), whereas shading maybe reduce the synthesis of the anthocyanins (Kubo et al. 1988). Similar findings were observed in this study. The anthocyanins synthesized quickly after seeds germination and so do PAL activity and soluble sugar content, and they show a positive correlation, as well as chlorophyll content.

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