Simultaneous induction of anthocyanin and peroxidase by sucrose in hypocotyls and roots of Chinese red radish seedlings

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Abstract

Anthocyanin and peroxidase (POD) are important active compounds in Chinese red radish (Raphanus sativus L.). The effects of exogenous sucrose, mixture of fructose and glucose (F/G 1:1), and mannose on anthocyanin accumulation and POD activity were investigated in hypocotyls and roots of red radish seedlings. Sucrose was most effective in inducing anthocyanin accumulation and POD activity in both organs, followed by F/G; mannose caused only a little increase of anthocyanin content and POD activity in hypocotyls, but a decrease in roots. The distribution of anthocyanin accumulation was different between hypocotyls and roots in the presence or absence of sucrose. Anthocyanin was clearly induced in root tips and cortex under exogenous sucrose. Accumulation of anthocyanin was closely correlated to the increase of endogenous sucrose content rather than glucose or fructose content. Sucrose significantly induced the expressions of late anthocyanin biosynthetic genes by up-regulating Raphanus sativus myeloblastosis 1 and Raphanus sativus transparent testa 8 in both organs. The POD activity significantly increased following the remarkable accumulation of anthocyanin in hypocotyls under exogenous sucrose, which may be partially required for anthocyanin oxidation. In roots, the high POD activity might lead to a low anthocyanin content by degrading anthocyanin and diverting the metabolic flux to lignin synthesis.

Additional key words: fructose, glucose, mannose, Raphanus sativus.

Introduction

Chinese red radish (Raphanus sativus L.) is an important root vegetable with green skin and red flesh. The red pigment in the root is predominantly pelargonidin, which is widely used as natural food colorant because of its bright pigment and color stability (Rodriguezsaona et al. 2010). Radish root, as a modified root, is composed of two anatomically distinct parts, the upper part originating from the hypocotyls and the lower part consisting of true root tissue (Takami and Kobayashi 2008). Hypocotyls of red radish have been known to accumulate anthocyanin in response to various environmental changes (Hara et al. 2003, Park et al. 2013, Su et al. 2016). However, the roots of red radish seedlings can be easily neglected because of no visible red color in the skin. In fact, the mature root of Chinese red radish is not only a rich source of anthocyanin but also peroxidase, both are important medicinal compounds (Curtis 2003, Wang et al. 2014). Anthocyanins is a class of flavonoids that have received significant attention due to their potential health benefits (He et al. 2010). The biosynthesis of anthocyanin is mediated by the phenylpropanoid pathway, which involves several enzymes such as phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), 4-coumarate-CoA ligase (4CL), chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), dihydrolflavonol 4-reductase (DFR), anthocyanidin synthase (ANS), and UDP-glucose:flavonoid 3-O-glucosyltransferase (UFGT). All of the genes encoding these enzymes have been identified in radish (Park et al. 2013). Members of three transcription factor families, repeat2repeat3-myeloblastosis (MYB), basic helix-loop-helix (bHLH), and WD40 repeat DNA protein (WDR), activate anthocyanin biosynthesis as components of the MBW complex (Hichri et al. 2011). The MYB and transparent
testa 8 (TT8) have been confirmed to be required for the activation of anthocyanin biosynthesis in red radish and purple cabbage (He et al. 2015, Lim et al. 2016).

Anthocyanins can be oxidized by peroxidases (PODs, EC 1.11.1.7) in vitro or in vivo (Wang et al. 2004, Zipor et al. 2015, Movahed et al. 2016). Peroxidase RsPrx1 purified from the roots of Chinese red radish can utilize pelargonidin as substrate in planta (Wang et al. 2004). Peroxidases, BcPrx01 and VviPrx31, are responsible for in planta degradation of anthocyanins in Brunfelsia calycina flowers and ripening grape berries under high temperature, respectively (Zipor et al. 2015, Movahed et al. 2016). Several studies have shown that peroxidase activity is high in plant tissues rich in anthocyanin. For example, POD activity was higher in purple-leaf cabbage than that in less pigmented genotype (Singh et al. 2010), and in red peel compared with the green one during nectarine maturation (Su et al. 2012). Our previous study showed the taproot of red radish had higher POD activity than that of white radish (Wang et al. 2014). In addition, the increase of POD activity was paralleled with anthocyanin accumulation during the development and senescence of Arabidopsis thaliana (Cosio et al. 2010), and in the peel during nectarine maturation (Su et al. 2012). High POD activity has been suggested to be required to degrade anthocyanin by controlling the rate of oxidation (Zhou et al. 2002). Therefore, POD activity seems to be correlated with anthocyanin content in plant tissues.

 Sugars can induce anthocyanin accumulation in several plant species (Hara et al. 2003, Solfanelli et al. 2006, Guo et al. 2011, Dai et al. 2014, Zhang et al. 2015). Different plant species showed distinct sensitivity to different sugars. Compared with glucose and fructose, sucrose has been proven more effective in inducing anthocyanin biosynthesis in Arabidopsis thaliana, broccoli seedlings, and radish hypocotyls (Hara et al. 2003, Solfanelli et al. 2006, Guo et al. 2011). In addition, the role of sucrose in anthocyanin induction could not be substituted by the mixture of glucose and fructose (1:1) in Arabidopsis and broccoli seedlings (Solfanelli et al. 2006, Guo et al. 2011). However, the reason for this phenomenon is unknown. In detached radish hypocotyls, sucrose enhanced anthocyanin production by inducing the expression of CHS and ANS (Hara et al. 2003). However, no reports have focused on the sugar-induced anthocyanin accumulation simultaneously in hypocotyls and roots of red radish seedlings. In the present study, both hypocotyls and roots of intact red radish seedlings were used for the first time to check the effect of sucrose, mixture of glucose/fructose (1:1), and mannose on the accumulation of anthocyanin. The endogenous soluble sugars and the transcripts of anthocyanin biosynthetic and regulatory genes were evaluated in both organs in response to different sugars. POD activity and isozymes were also determined during sugar-induced anthocyanin accumulation.

Materials and methods

Plants and treatments: Chinese red radish (Raphanus sativus L. cv. Man Tang Hong) seeds were ordered from Chinese Academy of Agricultural Sciences (Beijing, China). The seeds were immersed in 0.5 % (m/v) sodium hypochlorite solution for 30 min, rinsed with distilled water and then soaked in distilled water for 12 h. Seeds were germinated on filter paper moistened with distilled water for 1 d at 25 °C in darkness. Uniform gemmiparous seeds were selected and transferred to plastic boxes and cultivated in distilled water under a 16-h photoperiod, an irradiance of 160 µmol m⁻² s⁻¹, a temperature of 25 °C, and a relative humidity of 60 % in a growth chamber (PERCIVAL Company, Perry, IA, USA). Three-day-old seedlings were subjected to different treatments. Based on a previous study performed by Hara et al. (2003) on detached radish hypocotyls, 90 mM sugar was used to investigate anthocyanin accumulation and POD activity in red radish seedlings. Seedlings were treated with distilled water (control) or different sugars, 90 mM sucrose (Suc), 45 mM fructose + 45 mM glucose (F/G), 90 mM mannose (Man, an osmotic control) solution, respectively. The hypocotyl and root samples after 24 h treatment were frozen in liquid nitrogen and stored at -80 °C for RNA extraction. The hypocotyls and roots were collected 3 d later for measurement of hypocotyl and root length, soluble sugar content, anthocyanin content, and POD activity.

To investigate the dynamic changes of anthocyanin accumulation and POD activity, 3-day-old seedlings were treated with distilled water (control), 90 mM Suc and 90 mM Man solution for 5 d, respectively. The hypocotyls were collected at 12, 24, 48, 72, 96, and 120 h after sugar exposure for measurement of anthocyanin content and POD activity.

Observation of anthocyanin distribution in plant tissues: The roots were directly analyzed using a stereoscopic microscope (MI65C, Leica, Wetzlar, Germany). The cross section of hypocotyls and roots of radish seedlings were transected with a blade and then analyzed using a stereoscopic microscope.

Measurement of total anthocyanin content: Total anthocyanin content was measured according to the method described by Shin et al. (2013) with minor modification. Briefly, fresh hypocotyl and root samples (100 mg each) were ground and extracted in 600 mm² of methanol containing 1 % hydrochloric acid overnight at 4 °C. Subsequently, 200 mm³ of water and 200 mm³ of chloroform were added and the samples were centrifuged at 13 000 g for 10 min to deposit the sediment of the plant material. After centrifugation, the supernatant, the supernatant was separated and used to measure the absorbance at 530 nm and 657 nm using a spectrophotometer (TU-1901, Puxi, Beijing, China). One unit of anthocyanin was defined as one absorbance unit (A530 - 0.33 A657). All samples were measured as triplicates in three independent biological replicates.

Analysis of pelargonidin by high-performance liquid chromatography electrospray ionization tandem mass spectrometry: Extraction and purification of pelargonidin
were performed according to a previous report (Park et al. 2011). The extract was filtered through a 0.2 μm polytetrafluoroethylene syringe filter. The samples were then analyzed by a 1290 Series HPLC (Agilent Technologies, Palo Alto, CA), equipped with a variable wavelength detector. The results were analyzed by Agilent 1290 HPLC ChemStation software. The chromatographic separation was performed on a Zorbax Stablebond Analytical SB-C18 column (2.1 mm × 150 mm, 3.5 μm, Agilent Technologies, Rising Sun, MD). As mobile phase, 0.1 % formic acid in methanol (A) and 0.1 % formic acid in water (B) were used. Injection volume was 5 mm³, and flow rate was 0.3 cm² min⁻¹. The detection was at 250 nm, and the column oven temperature was set at 40 °C. The gradient program is described as follows: 0 - 1 min, 80 % B; 1 - 3 min, 80 - 0 % B; 3 - 5 min, 50 - 40 % B; 5 - 6 min, 40 % B; 6 - 6.1 min, 40 - 80 % B; 6.1 - 10 min, 80 % B. Pelargonidin-3-O-glucoside chloride (Extrasyntèse, Genay, France) was used as an external standard. Quantification of pelargonidin was calculated as pelargonidin-3-O-glucoside chloride equivalent. Obtained relative peak area was normalized by the external standard and fresh mass. Low-resolution electrospray mass spectrometry was performed with a Solarix ion trap mass spectrometer (Bruker Daltoniks, Billerica, MA, USA). The electrospray ionization conditions were as follows: ion spray 4.5 kV; curtain gas (137.9 kPa), nebulizing gas (379.2 kPa), and heating gas (413.7 kPa) were high-purity nitrogen (N₂); heating gas temperature 400 °C.

Measurement of soluble sugar content: Fresh hypocotyl and root tissues (100 mg, each) were collected to determine soluble sugar contents. Glucose, fructose, and sucrose content was measured using a commercially available sucrose, fructose, and glucose assay kits (Comin Biotechnology Co., Suzhou, China), respectively.

Extraction and analysis of POD activity: Fresh hypocotyls and roots (200 mg, each) were homogenized with 50 mM potassium phosphate buffer (pH 7.0) using a chilled mortar and pestle. The homogenate was centrifuged at 10 000 g at 4 °C for 20 min. The supernatant was used for the determination of POD activity as described previously (Wang et al. 2014). The reaction mixture contained 10 mM phosphate buffer (pH 7.0), 20 mM guaiacol, 40 mM H₂O₂, and enzyme extract in a total volume of 3 cm³. The reaction was initiated by adding H₂O₂ and the absorbance at 470 nm was measured at a 30 s interval for 3 min. One unit (U) of POD activity was defined as an absorbance change of 0.001 at 470 nm between the blank and the sample per min of reaction time.

Identification of POD isozymes: Protein was extracted from fresh hypocotyls and roots as crude POD enzymes, protein content was determined using Bradford method (Bradford 1976). Extracts containing 150 μg and 40 μg proteins from hypocotyls and roots, respectively, were separated on non-denaturing polyacrylamide gels (stacking gel 3 % (m/v) and separating gel 7.5 % (m/v)) as described by Laemmli (1970). After electrophoresis, the gel was incubated in a solution containing 2 mM benzidine. The reaction was initiated by adding 3 mM H₂O₂ and incubated at room temperature till brown bands appeared.

Extraction of RNA and real-time quantitative PCR: Total RNA was extracted from hypocotyl and root tissues using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. DNA-free total RNA (1 μg) was reverse transcribed into cDNA with oligo (dT) 20 primer and MMLV reverse transcriptase (Takara, Tokyo, Japan). The cDNA was diluted 10-fold for qPCR analysis.

Real-time PCR mixture (20 mm³ in total volume) included 10 mm³ of SYBR Premier Ex TaqII mix (Takara), 0.5 mm³ of each primer (10 μM), 2 mm³ of diluted cDNA, and 7 mm³ DNase-free water. Subsequently, the qPCR was performed on a LightCycler 96 real-time PCR instrument (Roche, Basel, Switzerland), initiated at 95 °C for 10 min, followed by 45 cycles at 95 °C for 10 s and 60 °C for 30 s. Melting curve analysis was performed at 95 °C for 10 s, 65 °C for 60 s, and 97 °C for 1 s. Amplification of RsACTIN2 gene was conducted as an internal control to normalize the expression of the target genes. The primers used for the qPCR analysis of anthocyanin biosynthetic and regulatory genes were designed based on the sequences from our previous study (Wang et al. 2014) and other reports (Park et al. 2013, Lim et al. 2016) on radish anthocyanin using the Primer3 software (http://primer3.sourceforge.net), and were listed in Table 1 Suppl. The relative expressions of the target genes were calculated according to the formula 2^(-ΔΔCT) (Livak and Schmittgen 2001). All analyses were repeated twice using three replicates.

Statistical analysis: Biological tri-replicates were used for each measurement and data was expressed as means ± standard error. Differences were determined by two-way ANOVA and the least significant difference test (SPSS version 17.0, SPSS Inc., Chicago, IL, USA) at a 0.05 level.

Results

To test the effect of different sugars on the growth of radish seedlings, the length of hypocotyl and root was measured at 3 d after treatment. As shown in Fig. 1, the elongation of hypocotyl and root was significantly suppressed by all three sugars. Hypocotyl length was reduced by 38, 34, and 41 % in seedlings treated with Suc, F/G, and Man, respectively (Fig. 1A). The root length of seedlings in the presence of Suc, F/G, and Man was 24, 20, and 35 % shorter than that of control seedlings (Fig. 1B).

It is clear that application of Suc or F/G significantly induced anthocyanin accumulation in both hypocotyls and roots, especially in lower hypocotyls and upper roots, which would develop into tuberous root later (Fig. 2A).

In contrast, same concentration (90 mM) of Man did not cause obvious anthocyanin accumulation in hypocotyls, but inhibited anthocyanin synthesis in roots (Fig. 2). This suggested that the induction of anthocyanin synthesis by
Suc and F/G was not due to osmotic stress. Obviously, Suc is more effective than its breakdown products, F/G, in inducing anthocyanin biosynthesis in both organs (Fig. 2). Suc treatment also promoted lateral root formation (Fig. 2B). The tips of primary root and lateral roots showed remarkable anthocyanin accumulation after Suc treatment, whereas only a little induction was observed after F/G treatment compared with controls, in which no visible anthocyanin was observed (Fig. 2B and Fig. 1 Suppl.). In hypocotyls, increased anthocyanin accumulation was
clearly observed in the epidermis and vascular cylinder, and in roots, anthocyanin was remarkably induced not only in the vascular cylinder but also in the cortex by Suc and F/G treatments, especially by Suc treatment (Fig. 2C,D).

We further quantified the content of anthocyanin and pelargonidin in radish. In the absence of sugars, hypocotyls accumulated more anthocyanin and pelargonidin than roots (Fig. 3). Compared with control, application of Suc, F/G, and Man increased total anthocyanin and pelargonidin content in hypocotyls by 271 and 523 %, 165 and 368 %, 64 and 124 %, respectively (Fig. 3). In roots, total anthocyanin and pelargonidin content was enhanced by 229 and 445 %, 128 and 214 % by Suc and F/G treatments, respectively, while decreased by 44 and 50 % by Man treatment, compared with control (Fig. 3). The quantification results were consistent with the visible color darkness.

Glucose, fructose, and sucrose are three major soluble sugar components in radish (Su et al. 2016). Under control condition, content of both glucose and fructose was significantly higher in hypocotyls than in roots, but no obvious difference in sucrose content (Fig. 4). In hypocotyls, different sugar treatments significantly increased the content of three soluble sugars (Fig. 4). In roots, the content of three soluble sugars was significantly enhanced by Suc and F/G treatments, while only glucose content was significantly increased by Man treatment (Fig. 4). Of all the three sugar treatments, Suc caused the largest increase in soluble sugars, followed by F/G and Man treatment. Compared with control samples, endogenous sucrose content in hypocotyls was increased by 324 and 180 %, and in roots, 596 and 199 % by Suc and F/G treatments, respectively (Fig. 4C). Under sugar treatments, hypocotyls still had higher glucose and fructose content than roots. In contrast, roots showed higher sucrose content than hypocotyls after Suc treatment (Fig. 4).

To understand the molecular mechanism of anthocyanin accumulation induced by sugar treatment, the expression of anthocyanin biosynthetic genes were examined by qPCR. As shown in Fig. 5, all anthocyanin biosynthetic genes were expressed in both organs under control conditions; compared with hypocotyls, roots showed a higher expressions of early biosynthetic genes (EBGs, RsPAL, RsC4H, and Rs4CL) but a lower expressions of late biosynthetic genes (LBGs, RsDFR, RsANS, and RsUFGT), which encode the key enzymes in the last steps of anthocyanin biosynthesis (He et al. 2015). The transcripts of all anthocyanin biosynthetic genes in both organs were significantly up-regulated by Suc and F/G treatments, but not by Man, which induced the expression of these genes in hypocotyls but inhibited them in roots. Sue treatment is more effective than F/G treatment in up-regulating the expression of anthocyanin biosynthetic genes in both organs, especially in hypocotyls. The expressions of

![Fig. 3. Effects of different exogenous sugars on content of total anthocyanin (A) and pelargonidin (B) in hypocotyls and roots of Chinese red radish seedlings. Means ± SEs, n = 35; different letters indicate significant differences at P < 0.05.](image)

![Fig. 4. Effects of different exogenous sugars on content of endogenous fructose (A), glucose (B), and sucrose (C) in hypocotyls and roots of Chinese red radish seedlings. Means ± SEs, n = 35; different letters indicate significant differences at P < 0.05.](image)
RsDFR, RsANS, and RsUFGT in hypocotyls treated with Suc were 4-, 5- and 4-fold higher than in those treated with F/G, respectively.

MYB and bHLH transcription factors play essential roles in activating anthocyanin biosynthetic genes among various plant species (Hichri et al. 2011). Therefore, transcripts of anthocyanin regulatory genes RsMYB1 and RsTT8 (bHLH) were also examined in both hypocotyls and roots of red radish seedlings. Compared with hypocotyls, roots showed higher expression of RsMYB1 but lower expression of RsTT8 under control condition (Fig. 5). Application of exogenous sugars significantly up-regulated the expressions of RsMYB1 and RsTT8 in both organs, with the exception of RsTT8 repressed in roots by Man. Similar to anthocyanin biosynthetic genes, Suc treatment induced the expression of RsMYB1 and RsTT8 more remarkably than F/G treatment in both organs, especially in hypocotyls. The expressions of RsMYB1 and RsTT8 in hypocotyls treated with Suc were 1.3- and 1.6-fold higher than those treated with F/G, respectively. RsTT8 was more sensitive than RsMYB1 to exogenous sugars. In hypocotyls, 6.7-fold up-regulation was detected for RsTT8 after Suc treatment while 3.3-fold up-regulation for RsMYB1.

Because PODs degrade anthocyanin (Zipor et al. 2015, Movahed et al. 2016), we checked total POD activity in red radish seedlings. Total POD activity was 8-fold higher in roots than that in hypocotyls under control condition (Fig. 6A). Application of Suc and F/G significantly enhanced total POD activity in both organs. In hypocotyls, total POD activities were increased by 258 and 176 % after treatment with Suc and F/G, respectively, compared with control samples. In roots, total POD activities were increased by 71 and 37 % after treatment with Suc and F/G, respectively. However, Man treatment resulted in no obvious change and a significant reduction in total POD activity.
activity in hypocotyls and roots, respectively. Generally, changes of POD activity corresponded with the changes of anthocyanin content caused by sugar treatments.

Since changes in enzyme activities may be reflected in qualitative changes in isozymes, peroxidase isozyme patterns were further investigated in both organs by native PAGE. Under control condition, two and six POD isozymes were detected in hypocotyls and roots, respectively (Fig. 6B). In hypocotyls, exogenous sugars except Man remarkably increased the activities of H3 and H4 isozymes, and induced two new isozymes (H1 and H2), especially H2. Man treatment also induced H1 and H2 isozymes, but remarkably decreased the activity of H3 isozyme. H1 and H2 isozymes might be associated with osmotic stress, but, sucrose can induce their expression. The activities of POD isozymes (R1 to R4) were significantly increased by Suc and F/G treatments, but decreased by Man treatment in roots, with the exception of R1. One new POD isozyme R5 was obviously induced in the roots by Suc and F/G treatments compared with controls.

To further understand the relationship between anthocyanin and POD, dynamic changes of anthocyanin accumulation and POD activity in hypocotyls treated withSuc and Man were examined. No obvious change was observed for anthocyanin accumulation in the control samples (Fig. 7A). Accumulation of anthocyanin was induced at 24 h after exposure to Suc, increased by 52 % from 24 to 48 h, and significantly increased from 72 to 120 h. Man also caused an increase of anthocyanin content but the effect was much weaker compared to Suc, especially from 72 to 120 h. Under control conditions, there was a slight increase of POD activity from 72 to 120 h (Fig. 7B). POD activity showed no obvious change from 12 to 48 h, but was significantly increased from 72 to 120 h after sucrose exposure. The increased POD activity by Man was observed only after 72 h, which was much lower compared to Suc. The results clearly indicate that POD activity was increased following the enhanced anthocyanin accumulation in radish seedlings under Suc treatment.

**Discussion**

Our study showed that exogenous sugars prompted anthocyanin accumulation not only in hypocotyls but also in roots of red radish seedlings (Figs. 2 and 3). This is consistent with previous studies in detached radish hypocotyls (Hara et al. 2003), grape berry, cut flowers of *Paonia suffruticosa*, whole broccoli sprouts, and *Arabidopsis* (Solfanelli et al. 2006, Guo et al. 2011, Dai
et al. 2014, Zhang et al. 2015). This is the first report showing an induction of anthocyanin in roots by exogenous sugars. Anthocyanin accumulation induced by exogenous Suc and F/G in red radish seedlings is not due to osmotic stress. This can be confirmed by the following results: 1) Suc, F/G, and Man treatments inhibited elongation of radish hypocotyls and roots, but resulted in significant difference in anthocyanin accumulation (Fig. 1 and 3); 2) Man, an osmotic control, caused a slight increase in anthocyanin content in hypocotyls and even a decrease in roots, while, notably anthocyanin accumulation was observed under Suc and F/G treatments (Fig. 3).

Sugar uptake and accumulation were found to be early events followed by the induction of anthocyanin in detached radish hypocotyls and Arabidopsis under exogenous sugars (Harra et al. 2003, Jeong et al. 2010). External sugars can be directly absorbed by plant organs such as leaves, roots, and berries, and further alter sugar metabolism in plant by regulating the activities of several related enzymes (Shin et al. 2013, Dai et al. 2014, Lobo et al. 2015). Sugar metabolism is complex because not only involves the interconversion of different sugars such as hexoses (glucose, fructose), sucrose, and starch, but also interacts with other metabolic processes (Lobo et al. 2015). In this study, the increased content of glucose, fructose, and sucrose in hypocotyls and roots by exogenous Suc and F/G may result from the uptake of exogenous sugars, transport, as well as the changes in sugar metabolism (Fig. 4).

Comparing the anthocyanin content and sugar content in red radish seedlings treated with different sugars, we found that anthocyanin content was notably correlated with endogenous sucrose content rather than with glucose or fructose content. This is based on these results (Figs. 3 and 4): 1) glucose and fructose content was significantly increased in hypocotyls by all of Suc, F/G, and Man treatments, but anthocyanin content was dramatically induced by Suc, and only slightly induced by Man; 2) anthocyanin content differences in hypocotyls between Suc and F/G, Suc, and Man treatments did not correlate with the glucose or fructose content difference in plants treated by Suc and F/G or Suc and Man, respectively; 3) glucose content was increased but anthocyanin content was reduced by Man treatments in roots. On the other hand, the most significant induction of anthocyanin in both hypocotyls and roots correlated well with the highest endogenous sucrose content induced by Suc treatment; the slight enhancement of anthocyanin by Man was accompanied with the small increase of sucrose in hypocotyls, and no induction of anthocyanin agreed with no change of sucrose in roots; the moderate anthocyanin content coordinated well with endogenous sucrose content induced by F/G treatment in radish. It has been proved that glucose can be converted into sucrose within a few hours and further induced anthocyanin accumulation in Arabidopsis (Solfanelli et al. 2006). The increased sucrose was found to stimulate anthocyanin biosynthesis in Arabidopsis loss-of-function mutants YDA and in the hypocotyls of radish sprouts (Su et al. 2016, Meng et al. 2018). Therefore, the positive correlation between sucrose content and anthocyanin accumulation is conserved in Brassicaceae family.

The hypocotyls of red radish always accumulated more anthocyanin than roots regardless of sucrose treatment or not, suggesting that hypocotyl is the major organ accumulating anthocyanin at seedling stage (Figs. 2 and 3). This may be due to the difference of anthocyanin biosynthesis and degradation in two organs. In the absence of Suc, anthocyanin accumulated at both the epidermis and vascular cylinder of hypocotyls, while only at the vascular cylinder in roots. Under exogenous Suc, significant induction of anthocyanin was observed at the tips of primary root and lateral roots, while, the accumulation of anthocyanin at the cortex and vascular cylinder of roots was much weaker compared with that at the epidermis and vascular cylinder of hypocotyls. These results suggest the capacity of anthocyanin biosynthesis in roots is lower than that in hypocotyls at seedling stage.

The mechanism modulating anthocyanin biosynthesis in radish is similar to that in Arabidopsis, i.e., EBGs can be activated by redundant MYB gene regulators, whereas, the activation of LBGs requires a ternary complex composed of MYB-hHLH-WD40 transcription factors (Petroni and Tonelli 2011). Regardless of Suc treatment, the higher expressions of LBGs and RsTT8, the more anthocyanin accumulation in hypocotyls (Figs. 3 and 5). In contrast, higher expression of EBGs and lower of LBGs in roots suggested that the precursors in the phenylpropanoid pathway were mainly used for the biosynthesis of other secondary metabolites but not anthocyanin (Fig. 5). It is known that radish roots contain lignin and various phenolic acids, which share the common precursors with anthocyanin (Park et al. 2013, 2016, Schäfer et al. 2016). The higher expression of EBGs resulted from the higher expression of RsMYB1 in roots (Fig. 5). It seemed that the lower expression of RsTT8 may lead to the lower expression of LBGs and further results in less accumulation of anthocyanin in roots. The repression of RsTT8 by Man resulted in the suppressed expression of LBGs, consequently, reduced anthocyanin content in roots (Fig. 5). These findings demonstrate that RsTT8 is essential for anthocyanin biosynthesis in radish as in other Brassica species (Mushtaq et al. 2016). Therefore, compared with F/G, Suc-induced higher accumulation of anthocyanin in hypocotyls and roots may result from the higher expression of LBGs, which correspondingly needed higher expressions of both RsMYB1 and RsTT8 as in broccoli sprouts and transgenic Petunia (Guo et al. 2011, Ai et al. 2016).

Anthocyanin can be oxidized to ensure appropriate content in plants. PODs can degrade anthocyanin in plants (Zipor et al. 2015, Movahed et al. 2016, Shigeto and Tsutsumi 2016). POD often occurs as multiple isozymes, which display organ-specific expression patterns and different sensitivity in response to exogenous stimulus (Passardi et al. 2005). Contrary to anthocyanin content, hypocotyls showed lower POD activity and fewer isozymes than roots regardless of the presence of Suc (Fig. 6). Thus, the higher accumulation of anthocyanin in hypocotyls may be due to not only higher biosynthesis but also lower oxidation by PODs. In both hypocotyls and
roots, the accumulation of anthocyanin induced by Suc or F/G was accompanied by increased POD activity. The parallel increase of POD activity and anthocyanin content was also observed during the development and senescence of Arabidopsis, and in the peel during nectarine maturation (Singh et al. 2010, Su et al. 2012). No increase or even a decrease of POD activity by Man suggested the increased POD activity by Suc and F/G was not due to osmotic stress but it was sugar specific. The comparison of dynamic changes in anthocyanin accumulation and POD activity showed that the increase of POD activity by Suc occurred after the induction of anthocyanin accumulation (Fig. 7), suggesting that the increased POD activity may be required to degrade anthocyanin by controlling the rate of oxidation (Zhou et al. 2002). Several sugar-specific POD isozymes (H1, H2, and R5) were detected in radish (Fig. 6B). Among them, H2 is the most active isozyme, suggesting its dominant role in the oxidation of anthocyanin under exogenous Suc.

Compared to hypocotyls, radish roots have lower anthocyanin content, but higher POD activity and more isozymes of POD regardless of the presence of Suc (Figs. 3 and 6). We speculated that the high activity of POD in roots might be required not only for the oxidation of anthocyanin but also for the development of root. Large amounts of lignin were accumulated in the roots during radish development (Feng et al. 2017). PODs participate in lignin formation by oxidizing soluble phenolics in many plant species (Marjamaa et al. 2009). In strawberry fruit, the induction of POD FaPRX27 could divert the metabolic flux from anthocyanin to lignin synthesis (Ring et al. 2013). It has been reported that there is a competition between anthocyanin and lignin biosynthesis for common precursors (Ring et al. 2013, Wang et al. 2016). These findings indicate that high activity of POD may lead to low content of anthocyanin not only by degrading anthocyanin but also prompting lignin biosynthesis, which competes with anthocyanin pathway enzymes for common precursors. Further work is needed to elucidate the roles of the sugar-induced specific POD isozymes in anthocyanin accumulation.

In conclusion, Suc was more effective than F/G in inducing anthocyanin accumulation in both hypocotyls and roots. Distribution pattern of anthocyanin was different in hypocotyls and roots. Anthocyanin accumulation in root tips and cortex was only observed under exogenous Suc and F/G. The sugar-induced anthocyanin accumulation was closely correlated with the increase of endogenous sucrose content rather than glucose or fructose content. LBGs, other than EBGs, are determinants for anthocyanin synthesis. POD functions not only to degrade anthocyanin by oxidation, but also to diverting the metabolic flux from anthocyanin to lignin synthesis. The high anthocyanin content in hypocotyls under Suc resulted from combination of high expression of LBGs, RsIT8, and low activity of PODs. While in roots, extremely high POD activity may not only degrade anthocyanin, but also divert the metabolic flux to lignin synthesis, leading to the low anthocyanin content.

References

Ai, T.N., Naing, A.H., Arun, M., Lim, S.H., Chang, K.K.: Sucrose-induced anthocyanin accumulation in vegetative tissue of petunia plants requires anthocyanin regulatory transcription factors. - Plant Sci. 252: 144-150, 2016.

Bradford, M.M.: A rapid method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. - Anal. Biochem. 72: 248-254, 1976.

Cosio, C., Dunand, C.: Transcriptome analysis of various flower and silique development stages indicates a set of class III peroxidase genes potentially involved in pod shattering in Arabidopsis thaliana. - BMC Genomics 11: 528, 2010.

Curtis, I.S.: The noble radish: past, present and future. - Trends Plant Sci. 8: 305-307, 2003.

Dai, Z.W., Meddar, M., Renaud, C., Merlin, I., Hilbert, G., Delrot, S., Gomès, E.: Long-term in vitro culture of grape berries and its application to assess the effects of sugar supply on anthocyanin accumulation. - J. exp. Bot. 65: 4665-4677, 2014.

Feng, H., Xu, L., Wang, Y., Tang, M., Zhu, X., Zhang, W., Sun, X., Nie, S., Muleke, E.M., Liu, L.: Identification of critical genes associated with lignin biosynthesis in radish (Raphanus sativus L.) by de novo transcriptome sequencing. - Mol. Genet. Genomics 292: 1151-1163, 2017.

Guo, R., Yuan, G., Wang, Q.: Sucrose enhances the accumulation of anthocyanins and glucosinolates in broccoli sprouts. - Food Chem. 129: 1080-1087, 2011.

Hara, M., Oki, K., Hoshino, K., Kuboi, T: Enhancement of anthocyanin biosynthesis by sugar in radish (Raphanus sativus) hypocotyl. - Plant Sci. 164: 259-265, 2003.

He, J., Giusti, M.M.: Anthocyanins: natural colorants with health-promoting properties. - Annu. Rev. Food Sci. Technol. 1: 163-187, 2010.

He, Q., Zhang, Z., Zhang, L.: Anthocyanin accumulation, antioxidant ability and stability, and a transcriptional analysis of anthocyanin biosynthesis in purple heading Chinese cabbage (Brassica rapa L. ssp. pekinensis). - J. Agr. Food Chem. 64: 132-145, 2015.

Hichri, I., Barrieu, F., Bogs, J., Kappel, C., Delrot, S., Lauvergeat, V.: Recent advances in the transcriptional regulation of the flavonoid biosynthetic pathway. - J. exp. Bot. 62: 2465-2483, 2011.

Jeong, S.W., Das, P.K., Jeoung, S.C., Song, J.Y., Lee, H.K., Kim, Y.K., Kim, W.J., Park, Y.I., Yoo, S.D., Choi, S.B., Choi, G., Park, Y.I.: Ethylene suppression of sugar-induced anthocyanin pigmentation in Arabidopsis. - Plant Physiol. 154: 1514-1531, 2010.

Laemmli, U.K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. - Nature 227: 680-685, 1970.

Lim, S.H., Song, J.H., Kim, D.H., Kim, J.K., Lee, J.Y., Kim, Y.M., Ha, S.H.: Activation of anthocyanin biosynthesis by expression of the radish R2K3-MYB transcription factor gene RsMYB1. - Plant Cell Rep. 35: 641-653, 2016.

Livak, K.J., Schmittgen, T.D.: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. - Methods 25: 402-408, 2001.

Lobo, A.K.M., Martins, M.O, Neto, M.C.L, Machado, E.C., Ribeiro, R.V., Silveira, J.A.G.: Exogenous sucrose supply changes sugar metabolism and reduces photosynthesis of sugarcane through the down-regulation of Rubisco abundance and activity. - J. Plant Physiol. 179: 113-121, 2015.

Marjamaa, K., Kukkola, E.M., Fagerstedt, K.V.: The role of xylem class III peroxidases in lignification. - J. exp. Bot. 60: 367-376, 2009.
Peroxidase as a candidate gene in Ipomoea batatas-expressed sweet potato ('Luoyang').

Rodriguez-Saona, L.E., Giusti, M.M., Wrolstad, R.E.: Color change mechanisms in model systems. - J. Food Sci. 64: 43-60, 2011.

Passardi, F., Cosio, C., Penel, C., Dunand, C.: Peroxidases have more functions than a Swiss army knife. - Plant Cell Rep. 24: 225-265, 2005.

Petroni, K., Tonelli, C.: Recent advances on the regulation of anthocyanin synthesis in reproductive organs. - Plant Sci. 181: 219-229, 2011.

Ring, L., Yeh, S.Y., Hübcherg, S., Hoffmann, T., Blanco-Portales, R., Fouch, M., Villatoro, C., Denoyes, B., Monfort, A., Caballero, J.L., Muñoz-Blanco, J., Gershenson, J., Schwab, W.: Metabolic interaction between anthocyanin and lignin biosynthesis is associated with peroxidase FaPRX27 in strawberry fruit. - J. Plant Physiol. 163: 43-60, 2013.

Rodriguez-Saona, L.E., Giusti, M.M., Wrolstad, R.E.: Color and pigment stability of red radish and red-fleshed potato anthocyanins in juice model systems. - J. Food Sci. 64: 451-456, 1999.

Schäfer, J., Brett, A., Trierweiler, B., Bunzel, M.: Characterization of cell wall composition of radish (Raphanus sativus L. var. sativus) and maturation related changes. - J. Agr. Food Chem. 64: 8625-8632, 2016.

Shigeto, J., Tsutsumi, Y.: Diverse functions and reactions of class III peroxidases. - New Phytol. 209: 1395-1402, 2016.

Shin, D.H., Choi, M.G., Lee, H.K., Cho, M., Choi, S.B., Choi, G., Park, Y.I.: Calcium dependent sucrose uptake links sugar signaling to anthocyanin biosynthesis in Arabidopsis. - Biochem. Biophys. Res. Commun. 430: 634-639, 2013.

Singh, B.K., Sharma, S.R., Singh, B.: Antioxidant enzymes in cabbage: variability and inheritance of superoxide dismutase, peroxidase and catalase. - Sci. Hort. 124: 9-13, 2010.

Solfanzelli, C., Poggi, A., Loreti, E., Alpi, A., Perata, P.: Sucrose-specific induction of the anthocyanin biosynthetic pathway in Arabidopsis. - Plant Physiol. 140: 637-646, 2006.

Su, M., Chen, K., Ye, Z., Zhang, B., Guo, J., Xu, C., Sun, C., Zhang, J., Li, X., Wu, A.: Physical changes and physiological characteristics of red and green peel during nectarine (cv. Hu018) maturation. - J. Sci. Food Agr. 92: 1448-1454, 2012.

Su, N., Wu, Q., Cui, J.: Increased sucrose in the hypocotyls of radish sprouts contributes to nitrogen deficiency-induced anthocyanin accumulation. - Front. Plant Sci. 7: 1976, 2016.

Takami, K., Kobayashi, S.: Mapping of QTLs controlling root shape and red pigmentation in radish, Raphanus sativus L. - Breed. Sci. 58: 55-61, 2008.

Wang, H., Yang, J., Min, Z., Fan, W., Firon, N., Pattanaik, S., Ling, Y., Peng, Z.: Altered phenylpropanoid metabolism in the maize Lc-expressed sweet potato (Ipomoea batatas) affects storage root development. - Sci. Rep. 6: 18645, 2016.

Wang, L., Burhennke, K., Kristensen, B.K., Rasmussen, S.K.: Purification and cloning of a Chinese red radish peroxidase that metabolise pelargonidin and forms a gene family in Brassicaceae. - Gene 343: 323-335, 2004.

Wang, L., Wang, Y., Wang, X., Li, Y., Peng, F., Wang, L.: Regulation of POD activity by pelargonidin during vegetative growth in radish (Raphanus sativus L.). - Sci. Hort. 174: 105-111, 2014.

Zhang, C., Fu, J., Wang, Y., Gao, S., Du, D., Wu, F., Guo, J., Dong, L.: Glucose supply improves petal coloration and anthocyanin biosynthesis in Paeonia suffruticosa ‘Luoyang Hong’ cut flowers. - Postharvest Biol. Technol. 101: 73-81, 2015.

Zhao, S., Sauve, R., Howard, E.: Identification of a cell wall peroxidase in red calli of Prunus incisa Thunb. - Plant Cell Rep. 21: 380-384, 2002.

Zipor, G., Duarte, P., Carqueijeiro, I., Shahar, L., Ovadia, R., Teper-Bammolker, P., Eshel, D., Levin, Y., Doron-Faigenboim, A., Sottomayor, M.: In planta anthocyanin degradation by a vacuolar class III peroxidase in Brunfelsia calycina flowers. - New Phytol. 205: 653-665, 2015.