

Expression and tissue and subcellular localization of anthocyanidin synthase (ANS) in grapevine

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Abstract Anthocyanidin synthase (ANS) is one of the key enzymes in the biosynthesis of both anthocyanins and proanthocyanidins in grapevine. Although substantial researches have investigated *ANS* gene expression and regulation at the transcriptional level, little is yet known about protein expression and distribution in grapevine. Here, the expression and tissue and subcellular localization of ANS in different Cabernet sauvignon grapevine tissues were investigated by using the techniques of Western blotting, immunohistochemical localization, immunoelectron microscopy, and confocal microscopy. The results showed that the ANS was expressed in the grape berries, leaves, stems, petioles, and leaf buds. In grape berry skin and flesh, ANS expression is developmental dependent. Immunohistochemical analysis revealed that ANS is primarily distributed in the exocarp, mesocarp, and seed of the fruit; in palisade and spongy tissues of the leaves; in the primary phloem and pith ray in the stems; and in the growth point and leaf primordium of the leaf buds. Furthermore, at the subcellular level, the ANS was mainly localized in the

cytoplasm regardless of cell types and some ANS were also found in the nucleus in the mesocarp vascular bundle and leaf bud cells. This research will give further insight for the biosynthesis and regulation of different flavonoid compounds in grapevine.

Keywords Anthocyanidin synthase · Grapevine · Tissue localization · Subcellular localization · Transient expression

Abbreviations

ANR	Anthocyanidin reductase
ANS	Anthocyanidin synthase
BSA	Bovine serum albumin
F3'5'H	Flavonoid 3', 5'-hydroxylase
IgG	Immunoglobulin fractions
PAs	Proanthocyanidins
PBS	Phosphate-buffered saline
RSR	Regular salt rinse solution

Introduction

Anthocyanins and proanthocyanidins (PAs) are the two major flavonoids involved in many plant functions. In grapevine, these compounds play important roles against UV irradiation and microbial and fungal infections in vegetative tissues (Chamkha et al. 2003; Harborne and Williams 2000) and help determine the color, astringency, and quality of grape berries and wine (Bogs et al. 2005; Chamkha et al. 2003). Furthermore, they can also act as antioxidants with beneficial effects for human health including protection against free radical-mediated injury and cardiovascular disease (Halliwell et al. 2005). So, the biosynthesis and regulation of anthocyanins and PAs in

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grapevine have attracted more and more attention over the past several years.

Common steps are shared in the biosynthesis of PAs and anthocyanins in the flavonoid pathway, in which anthocyanidin synthase (ANS; EC 1.14.11.19), one of the key enzymes at the branch point, converts the colorless leucoanthocyanidins to the colored anthocyanidins (Abrahams et al. 2003; Saito et al. 1999; Bogs et al. 2007). In the past years, the ANS gene has been cloned and characterized in many plants (Nakajima et al. 2001; Saito et al. 1999; Shimada et al. 2005; Turnbull et al. 2004). It is regulated developmentally in specific tissues or cell types and affected by stress (Bogs et al. 2005, 2007; Castellarin et al. 2007; Gollop et al. 2001; Deytieux et al. 2007). In Shiraz and Gamay grapevine, the transcript of ANS gene has been shown to be expressed in almost all the plant organs (Boss et al. 1996a, b; Gollop et al. 2001). However, little is yet known of the expression and regulation of ANS at the protein level in grapevine.

Evidence from the red form of *Perilla frutescens* has supported the fact that ANS is localized in the epidermal cells corresponding to the epidermis-specific accumulation pattern of anthocyanins (Saito and Yamazaki 2002). Previous studies from other vegetative tissues of plants have also suggested that the flavonoid biosynthetic enzymes are localized in the epidermal cells (Saslowky and Winkel-Shirley 2001; Saslowky et al. 2005). It is known that the accumulation of PAs and anthocyanins is tissue-specific in grapevine (Boss et al. 1996a, b). Anthocyanins are primarily accumulated in berries, and PAs are present in the leaves, tendrils, flowers, root, and berries of grapevine (Boss et al. 1996b). It is therefore necessary to further investigate the tissue distribution and localization of corresponding enzymes including ANS.

At the subcellular level, Pang et al. (2007) have found that *Medicago* ANS was localized in the cytosol. Substantial researches of vegetative tissues have demonstrated that flavonoids are biosynthesized in the cytoplasm (Hrazdina and Jensen 1992; Jorgensen et al. 2005; Saslowky and Winkel-Shirley 2001; Winkel-Shirley 1999), which were transported to the vacuole for accumulation (Gomez et al. 2009; Grotewold and Davies 2008; Kitamura et al. 2004; Poustka et al. 2007; Zhao and Dixon 2010). Some flavonoids and flavonoid enzymes have also been found in the nucleus (Feucht et al. 2004; Polster et al. 2006; Saslowky et al. 2005; Naoumkina et al. 2007; Yu et al. 2008). The subcellular localization of flavonoid biosynthesis in different grapevine tissues is unclear. Determining the subcellular localization of ANS is very important for understanding the sites of flavonoid metabolism in grapevine.

In this study, the accumulation of PAs and anthocyanins in different grapevine tissues was determined. The expression of ANS and its tissue and subcellular distribution

patterns in different grapevine tissues were further studied with Western blotting, immunohistochemical localization, immuno-electron microscopy, and confocal microscopy techniques. The objectives of this study were to increase our understanding of flavonoid biosynthesis mechanism and provide additional insights into engineering this metabolic pathway in grapevine.

Materials and methods

Materials

Grape berries (*Vitis vinifera* L. cv. Cabernet Sauvignon) were collected from a commercial vineyard during the 2006 and 2007 seasons in the western suburbs of Beijing, People's Republic of China. Sampling took place per 10 days from 20 days after full bloom to complete ripening (about 120 days after full bloom) according to the method described by Chen et al. (2006). The samples were transported to the laboratory, where the freshly harvested berries were randomly selected on the basis of similar size and the absence of physical injuries or infections. Once separated, the fresh berries were immediately peeled by hands to obtain the skin and flesh samples and then were either embedded for localization of ANS or quickly frozen in liquid nitrogen. These samples were stored at -80°C pending further analysis.

The vegetative tissues used throughout the experiments were derived from 1-year-old potted grapevines of *V. vinifera* L. cv. Cabernet Sauvignon plants. The plants were raised in a greenhouse at 25°C with 16 h light/8 h dark photoperiod and 65% relative humidity. Plants with uniform vegetative growth were selected for the study, and the experiment was initiated when the plants were with 8~10 functional leaves (Supplementary Fig. 1). The different plant parts were cut as shown in Supplementary Fig. 1 and were either embedded for localization of ANS or stored at -80°C after freezing in liquid nitrogen until further analysis.

All chemicals were purchased from Sigma unless otherwise noted. The polyclonal antibody against grape ANS was prepared in our laboratory (Wang et al. 2010). The purified anti-ANS immunoglobulin (IgG) was used in the following experiments.

Quantification of total anthocyanins and PAs

Total anthocyanins of the developing grape berry skins were extracted according to the method of Boss et al. (1996a). The content was determined using the pH-differential method described by Orak (2007). Total anthocyanin contents of the vegetative tissues of young

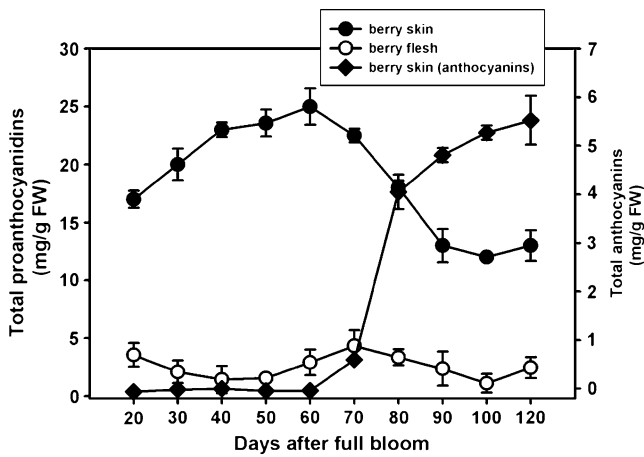


Fig. 1 Changes of anthocyanin and PA content in the skin and flesh of grape berries (*Vitis vinifera* L. cv. Carbernet Sauvignon) sampled at different developmental stages

grape plants were determined using the methods described by Sheoran et al. (2006) and Zhang et al. (2002). The anthocyanin contents were represented as color value (CV per gram fresh weight).

Total PAs were extracted according to Kennedy et al. (2000) with minor modifications. Tissue samples (0.5 g) were extracted twice with 20 ml 70% acetone solution (containing 0.1% Vc) for 6 h at 25°C in the dark with occasional shaking. After centrifugation at 8,000 rpm for 15 min, the combined supernatants were condensed by rotary evaporation, and then added ddH₂O to a total volume of 50 ml. The concentration of PAs was determined by the method of Reddy et al. (2007) using procyanidins as standard.

RNA preparation and RT-PCR analysis

Total RNA was isolated from the different tissues with the method described by Wen et al. (2005). The complementary DNAs (cDNAs) were synthesized according to the manufacturer’s instruction of AMV reverse transcriptase (Promega A3500).

The messenger RNA (mRNA) expression patterns of *ANS* and *Actin1* were examined by RT-PCR. The amplification of *Actin1* cDNA was used as an internal control. According to

the published sequences of grape *ANS* and *Actin1* (GenBank accession no: EU156063 and AY680701), gene-specific primers for *ANS* (forward: 5'-AGGGCCTCAGGTTCCAAC TAT-3'; reverse: 5'-CTCCCACTCAAGCTGTCCACT-3') and *Actin1* (forward: 5'-GATTCTGGTGATGGTGTGAGT-3'; reverse: 5'-GACAATTTCCCGTTCAGCAGT-3') were selected for expression analysis. The expected sizes of the PCR products were 292 and 168 bp, respectively. For each primer set, PCR conditions were optimized for T_m using gradient PCR and optimized for the number of cycles by running the above PCR for 36 cycles and removing the reaction product every third cycle from cycles 15 to 36 to obtain the following cycling parameters: *ANS*, 26 cycles, T_m 50°C; *Actin1*, 29 cycles, T_m 47°C. The amplified products were separated on 1.5% agarose gel and analyzed with Gene analysis software package (Gene Company). For each sample, the RNA was extracted twice and RT-PCR was performed three times. The identity of all PCR products was confirmed by sequencing analysis at the Beijing Sunbiotech Company (Beijing, China).

Protein extraction and Western blotting analysis

Total proteins were extracted according to the method of Famiani et al. (2000). Sodium dodecyl sulfate polyacrylamide gel electrophoresis was carried out using 8% polyacrylamide gels according to Laemmli (1970). The total proteins (2 μg) were loaded per well. Here, it should be noted that these immunoblotting analyses were based on the same amount of loading proteins in each sample. After electrophoresis, the proteins were elector-transferred to nitrocellulose membranes (0.45 μm, Amersham Life Science) using a transfer apparatus (Bio-Rad) technique described by Isla et al. (1998). Immunological detection of proteins on the NC membrane was carried out using a primary polyclonal ANS antibody in a 1/1,000 dilution at 25°C with an alkaline phosphatase-conjugated anti-rabbit IgG antibody from a goat (1/800 dilution) as a secondary antibody (Sigma-Aldrich). The membrane was stained with 10 ml of 5-bromo-4-chloro-3-indolyl phosphate/nitro blue terazolium (Promega) in the dark, and the reaction was terminated by the addition of

Table 1 Concentration of proanthocyanidins and anthocyanins in different grapevine tissues

Grapevine tissues	Proanthocyanidins (mg/g fresh weight)	Anthocyanins (CV/g fresh weight)
Stem tip	41.1	0.19
Stem P	32.9	0.90
Stem X	6.5	n/d
Leaves	36.6	n/d
Leaf bud	81.0	0.55
Petiole	18.0	0.51
Root	20.9	n/d

n/d anthocyanins not detected

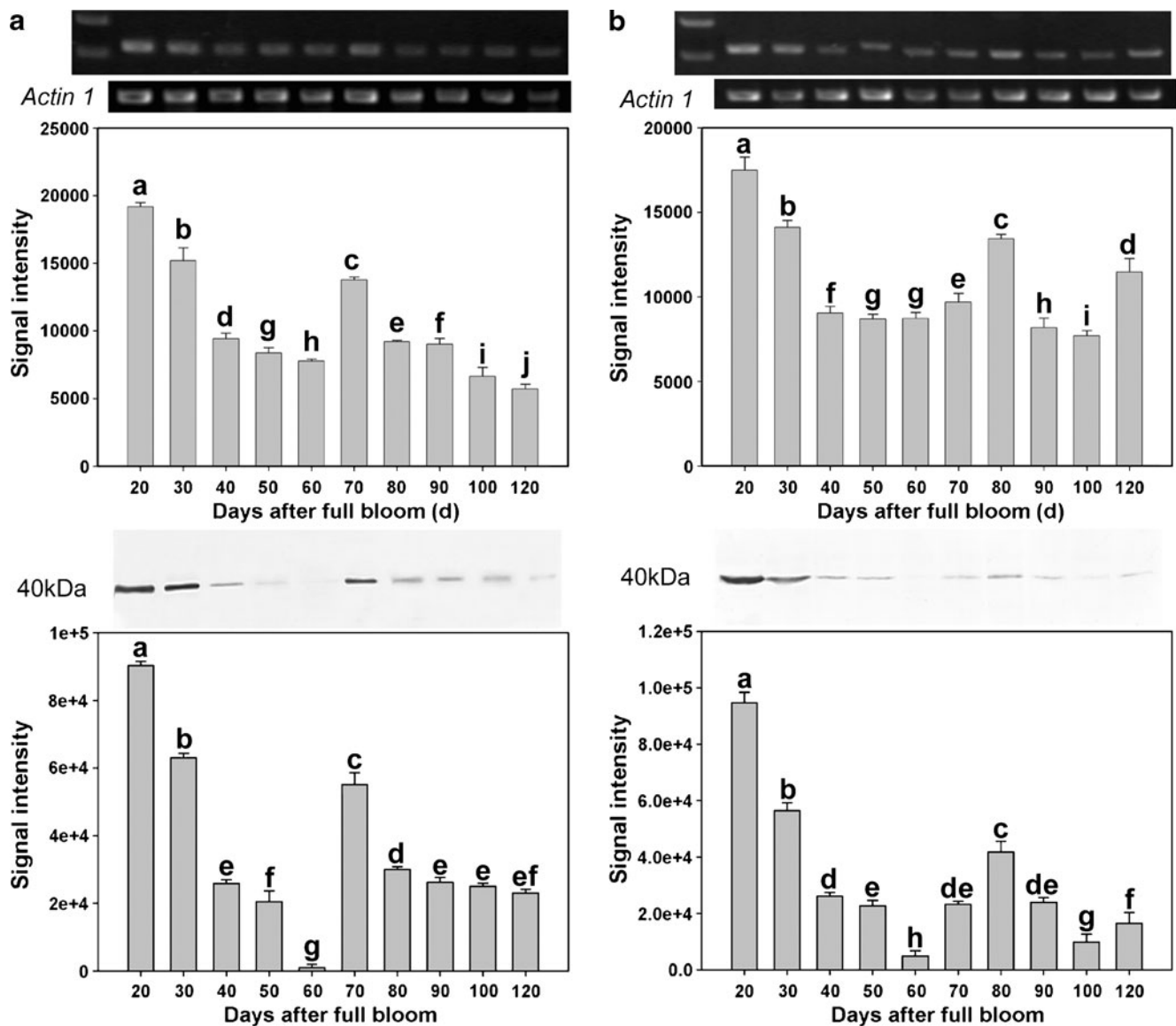


Fig. 2 The expression of ANS protein and ANS gene transcript accumulation in the skin and flesh of developing grape berries. **a** Changes of expression of ANS protein and transcript accumulation of *ANS* gene in developing grape berry skins. Fragment of 292 bp (*ANS*) was amplified. As control, fragment of *actin 1* (168 bp) was amplified simultaneously. Immunodetection of ANS in the soluble crude extracts throughout the berry development showed that only one band of polypeptide was immunodetected (about 40 kDa). The identical

amount of protein (2 μ g) was loaded per lane. **b** Changes of ANS protein and *ANS* gene transcript accumulation in berry flesh during grape berry development. The data of signal intensity in **a** and **b** are means from three analyses of one of three independent replicates. Bars are standard errors ($n=3$). Different letters indicate a statistical difference at $P \leq 0.05$ among samples according to Duncan's multiple range tests

double-distilled water. The amount of ANS protein was quantified by scanning the nitrocellulose after immunoblotting with a densitometer using the ImageQuant software. For each sample, the total protein was extracted twice and Western blotting was performed three times.

Immunohistochemical localization of ANS

The process of ANS immunohistochemical localization was done essentially as described previously (Hou and Huang

2005) with some modifications. After the grape tissues were cut, they were fixed overnight in a solution containing 4% paraformaldehyde and 2.5% glutaraldehyde at 4°C, dehydrated with a graded ethanol series, embedded in paraffin, and sectioned into slices. The slides were spread with polylysine before the fixing of the sections. Dried sections were deparaffinized with xylene, hydrated in an ethanol–water series, and then washed by 10 mM phosphate-buffered saline (PBS, pH 7.0, containing 0.2 g/l KCl, 2.19 g/l $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 0.482 g/l KH_2PO_4) for

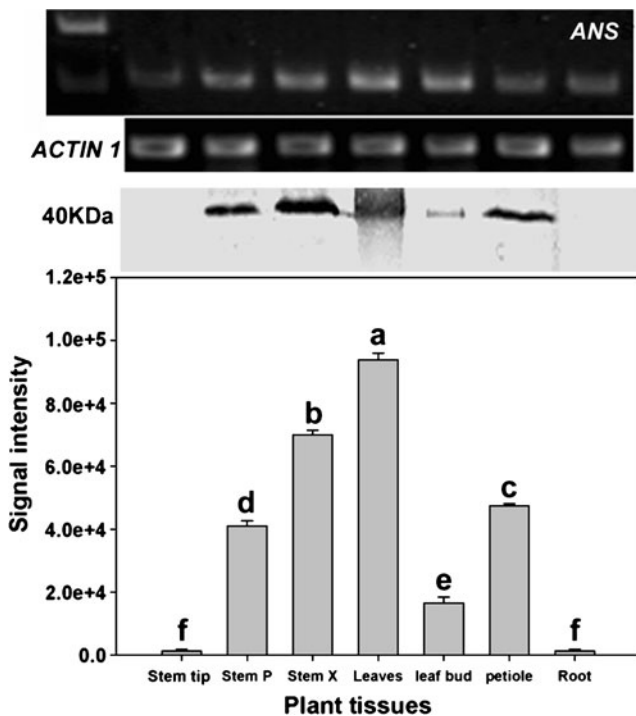


Fig. 3 The accumulation of *ANS* gene transcript and ANS protein in different grapevine vegetative tissues. The *upper artwork* shows the RT-PCR result of ANS. The *bottom artwork* shows Western blotting analysis of ANS in different tissues of young grape plants. The data of signal intensity are means from three analyses of one of two independent replicates. Bars are standard errors ($n=3$). Different letters indicate a statistical difference at $P \leq 0.05$ among samples according to Duncan's multiple range tests

5 min. Slides were blocked for 45 min at room temperature in a blocking solution [10 mM PBS, 0.1% (v/v) Tween-20, 1.5% (w/v) glycine, 5% (w/v) bovine serum albumin (BSA)], then rinsed in a regular salt rinse solution [RSR, 10 mM PBS, 0.1% (v/v) Tween-20, 0.8% (w/v) BSA, 0.88% (w/v) NaCl] for 5 min, and washed briefly with 10 mM PBS supplemented with 0.8% (w/v) BSA (PB) to remove Tween-20. 100 μ l primary ANS antibody was added to each slide and incubated at 4°C overnight in a humidity chamber. They were washed vigorously twice in a high-salt rinse solution [10 mM PBS, 0.1% (v/v) Tween-20, 0.1% (w/v) BSA, 2.9% (w/v) NaCl] followed by a 10-min wash with RSR and a brief rinse with 10 mM PBS. Then, 100- μ l secondary antibodies were added to the slides [1:100 (v/v)] with dilution of the anti-rabbit IgG-alkaline phosphatase conjugate (1 mg/l Promega) and incubated at room temperature in a humidity chamber. After rinsing twice in RSR and once in water, the slides were developed for approximately 15 min by adding 200 μ l of Western Blue stabilized substrate for alkaline phosphatase. As the blue/green color was observed on the sections, they were rinsed with water, dehydrated, and mounted with a cover glass for photographing.

To verify the reliability of the immunolocalization technique and the specificity of the antibody, two types of negative controls were set up. In the first one, the antibody was omitted to test possible unspecific labeling of the goat anti-rabbit IgG-alkaline phosphatase conjugate. In the second one, rabbit preimmune serum was used instead of the rabbit antibody to test the specificity of the antibody. The other procedure of immunolocalization process was preceded as usual. The control experiments were performed more than three times for each sample.

Subcellular immunogold labeling of ANS

Tissue preparation was essentially conducted according to the methods as previously described (Chen et al. 2006). The immunostaining procedure followed that of Ruelland et al. (2003). The ultrathin sections were incubated with TBSTG buffer (100 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% Triton X-100, 3% (w/v) BSA) for 30 min and then crossed directly with affinity-purified IgG to ANS antibody in TBSTG. Following washing three times for 5 min each with TBSTG buffer, the sections were incubated for 1 h with a goat anti-rabbit IgG conjugated to 10-nm gold particles at 1:100 dilutions in the TBSTG buffer at room temperature. The sections were rinsed consecutively with TBSTG and double-distilled water, then stained with 2% uranyl acetate and 4% lead citrate. The ultrathin sections were examined with a JEM-100S electron microscope.

Two types of control were performed in order to test the specificity and reliability of the immunogold labeling. The first one was omission of the antiserum to test the possible unspecific labeling of goat anti-rabbit IgG antibody-gold conjugate, and the second one was the use of the rabbit preimmune serum instead of the rabbit antiserum (incubation of ultrathin sections in preimmune serum instead of antiserum before immunogold labeling) to test the specificity of the antibody. More than three repetitions of the control experiments were performed for each sample.

Transient expression of ANS-GFP in *Arabidopsis* protoplasts

For observation of the subcellular localization of ANS in living cells, the full-length ORF of ANS cDNA was PCR-amplified by using primers 5'-CGCTCGAGATGGTACTTCAGTGG-3' (forward) and 5'-CCGGAATTCTAGATTTGGAGAGTAGAGC-3' (reverse). The PCR product was then fused to the upstream of the enhanced green fluorescent protein (GFP; Cormack et al. 1996) at the *Xho*I (5' end)/*Eco*RI (3' end) sites in the cauliflower mosaic virus 35S-EGFP-Ocs 3' vector

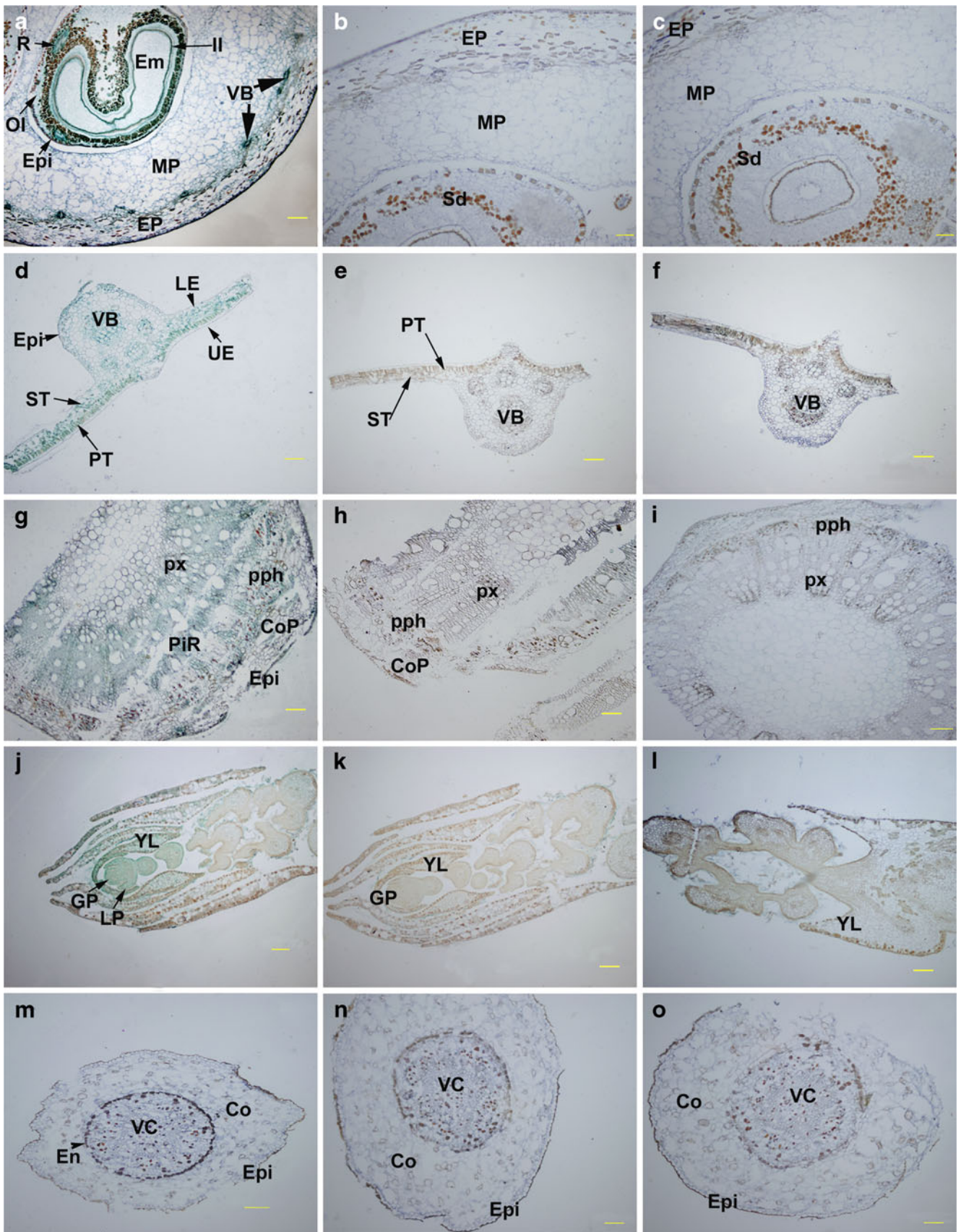


Fig. 4 Immunohistochemical localization of ANS in different organs of grapevine. **a** The distribution of ANS in grape berries; **d** tissue localization of ANS in grape leaves (mesophyll, vein); **g** immunohistochemical localization of ANS in grapevine stems; **j** immunohistochemical localization of ANS in grapevine leaf buds; **m** tissue localization of ANS in grapevine roots. **b–c, e–f, h–i, n–o** Negative controls of immunohistochemical localization of ANS in grapevine berries, leaves, stems, leaf buds, and roots, respectively. **b, e, h, n** Without the primary ANS antibody; **c, f, i, o** the primary ANS antibody was substituted with the preimmune serum of rabbit. No ANS signal was observed in these controls. *EP* exocarp, *MP* mesocarp, *VB* vascular bundle, *Epi* epidermis, *OI* outer integument, *II* inner integument, *R* raphe, *Em* endosperm, *Sd* seed, *UE* upper epidermis, *LE* lower epidermis, *PT* palisade tissue, *ST* spongy tissue, *PPh* primary phloem, *PX* protoxylem, *PiR* pith ray, *CoP* cortex parenchyma cells, *GP* growth point, *LP* leaf primordium, *YL* young leaf, *Co* cortex, *En* endoderm, *Vc* vascular cylinder. *Bar*=10 μ m

(pEZS-NL vector, Dr. Ehrhardt, <http://deepgreen.stanford.edu>). This vector does not express GFP well without adding coding sequence to the 5' end of the ORF of GFP; thus, the control cells do not show fluorescence of GFP. Protoplasts were isolated from the leaves of 3- to 4-week-old plants of *Arabidopsis* (ecotype Columbia) and transiently transformed using PEG essentially according to Ueda et al. (2001). Fluorescence of GFP was observed by a confocal laser scanning microscope (Bio-Rad MRC 1024) after incubation at 23°C for 16 h.

Results

Total anthocyanin and PA contents in different grapevine tissues

In grape skin, anthocyanins were first detected in berries sampled at 70 days after full bloom (Fig. 1), and we considered that this sampling date most represented veraison stage (the onset of ripening) (see Wang et al. 2010; Fig. 3). Following veraison, a substantial increase was observed in anthocyanin levels up to harvest (120 days after full bloom). No anthocyanins were detected in grape berry flesh. The accumulation of PAs in grape skin (Fig. 1) occurred at early development stage, reaching a maximum at 60 days after full bloom, and then declining during ripening. A small amount of PAs was observed in grape berry flesh during grape berry development.

In order to investigate the distribution of anthocyanins and PAs in grapevine vegetative tissues, seven different tissues from young grape plants were selected including the leaves, stems (stem phloem and stem xylem), petioles, leaf buds, stem tips, and roots (Supplementary Fig. 1). As shown in Table 1, anthocyanins were accumulated in the stem tips, stem phloem, leaf buds, and petioles. The

maximum concentration of anthocyanins was found in stem P (stem phloem), followed by leaf buds and petioles; the minimum concentration was found in stem tips. There were no anthocyanins detected in other sampled tissues. PAs were detected in all of the sampled tissues. The concentration of PAs in the various organs of young grape plants ranged from 6.5 mg/g FW (fresh weight) to 81.0 mg/g FW. Leaf buds contained the largest amount of PAs (81.0 mg/g FW); the stem tips (41.1 mg/g FW), leaves (36.6 mg/g FW), stem P (32.9 mg/g FW), roots (20.9 mg/g FW), and petioles (18.0 mg/g FW) showed lower concentration; the minimum was found in the stem xylem (6.5 mg/g FW).

The expression of ANS in developing grape berry skin and flesh

As shown in Fig. 2a, the changes of the ANS protein amounts and *ANS* gene transcript accumulation in developing grape berry skins were detected by Western blotting and RT-PCR. A single polypeptide with molecular mass of approximately 40 kDa was detected specifically from the protein fractions of the grape berry skins (Fig. 2a). The maximum accumulation of the ANS protein and *ANS* gene transcript occurred at early stage (20 days after full bloom) and then decreased gradually 30 to 60 days after full bloom. Following this period, the ANS amounts increased rapidly at veraison (70 days after full bloom), then decreased again during the later part of ripening stage.

Changes in ANS accumulation in grape berry flesh samples showed similar pattern to that in skins (Fig. 2b). The ANS protein and *ANS* gene transcript showed maximum accumulation at 20 days after full bloom. There was then a reduction during the successive development stage. Following this period, a coordinate increase was observed in accumulation of the ANS protein and *ANS* gene transcript in the sample (70 days after full bloom) at approximately the time of veraison. The accumulation reached to a higher level at 80 days after full bloom, then decreased throughout the remainder of berry development.

The expression of ANS in grapevine vegetative tissues

As shown in Fig. 3, the maximum of transcript accumulation was found in the leaves. The lower level of *ANS* gene transcript accumulation was found in other tissues, and the minimum of that was investigated in stem tip and roots. The ANS protein was accumulated in leaves, stems, petioles, and leaf buds. The immune signal level in leaves and stems reached the maximum, and a significant accumulation of ANS protein was also detected in the petioles and leaf buds. There was no ANS detected in stem tip and roots.

Immunohistochemical localization of ANS in different grapevine tissues

In order to observe the exact tissue localization of ANS in grapevine, five tissues were used including grape berry, leaves, stems, leaf buds, and roots. Endogenous ANS was immunohistochemically localized in grapevine tissues using the ANS polyclonal antibody (Fig. 4a–o). The blue-green color is the ANS immune signal, representing the natural distribution of endogenous ANS in the grapevine tissues. The results in Fig. 4a–c, d–f, g–i, j–l, and m–o showed the transverse sections of the grapevine tissues—grape berry, leaves, stems, leaf buds, and roots, respectively. Because maximum ANS protein was detected in grape berries at early stage, the grape berries at 20 days after full bloom were used. As shown in Fig. 4a, strong ANS signals were detected in the grape seed, mesocarp vascular bundles, and exocarp, while signals were also localized in mesocarp parenchyma cells. In grape seed (Fig. 4a), strong ANS signals were observed in epidermis, outer integument, inner integument, and the cells around the raphe. Slight ANS signals were distributed in medium integument and little ANS signals were investigated in the endosperm. In the grape leaves (Fig. 4d), intensive ANS signals were found in mesophyll and vein. In mesophyll (Fig. 4d), the ANS signals were mainly present in palisade and spongy tissue, while little ANS signals were investigated in the upper and lower epidermis. In leaf vein (Fig. 4d), the ANS signals were mainly localized in the vascular bundles, and obvious ANS signals were also found in the epidermis. In the stem (Fig. 4g), the ANS signals were mainly localized in the primary phloem and pith ray, and there were signals present in the epidermis of the stems, too. In leaf buds (Fig. 4j), the obvious ANS signals were detected in the growth point, leaf primordium, and young leaves. There was no obvious ANS signal in the grapevine roots (Fig. 4m).

Additionally, little ANS signal was found in the controls without the antiserum (Fig. 4b, e, h, k, n), or in any of the preimmune serum controls (Fig. 4c, f, i, l, o), indicating that the antibody was specific and the unspecific signal was negligible.

Subcellular localization of ANS in grapevine

In order to determine the subcellular localization of ANS in grapevine, four different tissues were chosen to detect the subcellular distribution of ANS in grapevine, including the vascular tissue in the mesocarp of grape berry, the leaves, stems, and leaf buds. The subcellular localization of ANS in the mesocarp vascular tissue cells was shown in Fig. 5. In the sieve element (Fig. 5a, b), the gold particles representing ANS were mainly localized in the cytoplasm, and no other organelles were observed in the cells. For the companion cells (Fig. 5c, d), the cytoplasm was heavily

labeled by gold particles; a few particles were also localized in the nucleus.

The subcellular localization of ANS in grapevine leaves was shown in Fig. 6. ANS visualized by gold particles were mainly localized in the cytoplasm. The cytoplasm localization of ANS in leaves was confirmed by the transient expression of ANS–GFP in the mesophyll protoplast of *Arabidopsis* (Supplementary Fig. 2). In the stem cells (Fig. 7a, b), gold particles representing ANS were also primarily found in the cytoplasm; few particles were localized in other organelles. For the leaf bud cells (Fig. 7c–e), the gold particles representing ANS were mainly localized in the cytoplasm and nucleus.

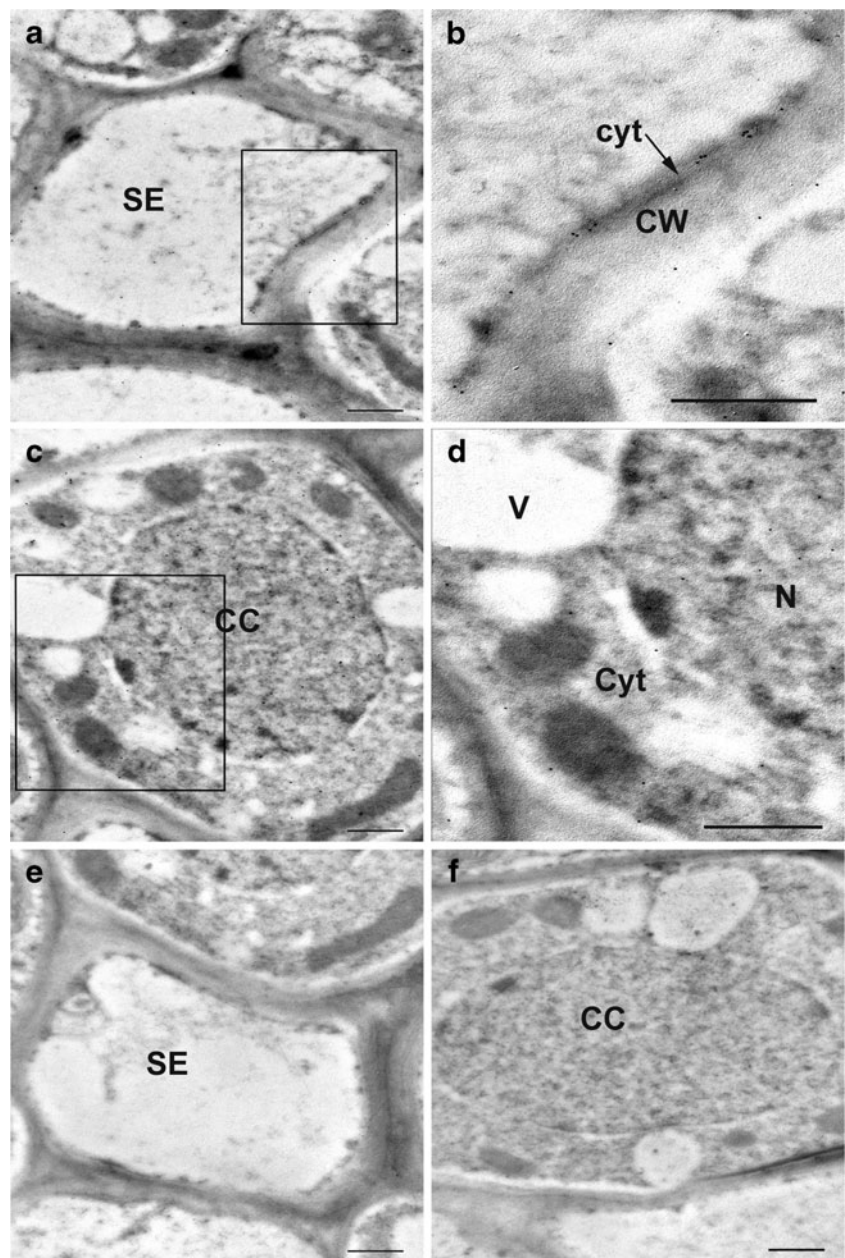
Substantially, no gold particles were found in the controls without the antibody (Figs. 5e and 6d) and in any of the preimmune serum controls (Figs. 5f and 6e), indicating that the antibodies were specific and unspecific labeling was negligible.

Discussion

The ANS gene has been detected to be expressed in almost all the organs of Shiraz grapevine: berry skin, berry flesh, seed, leaves, tendril, and green cane (Boss et al. 1996a, b). Gollop et al. (2001) have also found that the ANS promoter fusion was expressed in the leaves, meristem tissues, stems, and root of Gamay grapevine plants. Our present results from Cabernet Sauvignon grapevine revealed that the ANS protein was accumulated in the berry skin, flesh, seed, leaves, stems (stem P and stem X), petioles, and leaf buds, but not in the stem tip and root. Boss et al. (1996b) have suggested that the expression of flavonoid biosynthesis genes varied according to the cultivar. It indicates that the differences in the patterns of ANS expression in Cabernet Sauvignon, Shiraz, and Gamay may be due to varietal influences. Charrier et al. (1998) have also observed the difference of flavanone 3-hydroxylase expression and flavonoid localization in the nodules of three legume plants. In berry skin and flesh, it was noteworthy that the expression of the ANS gene and protein is developmental-dependent (Fig. 2), which is consistent with previous studies (Boss et al. 1996a, 2005, 2007; Deytieux et al. 2007), and the ANS was subjected to both transcriptional and translational regulation. In grape skin, the expression of ANS corresponds well to the accumulation of PAs and anthocyanins. However, no anthocyanins and a small amount of PAs were detected in berry flesh. It might be that the PAs synthesized in fruit flesh were transported to the grape berries skin or some other tissues to accumulate (Braidot et al. 2008a).

As to ANS expression in grapevine vegetative tissues, a large amount of ANS protein and ANS gene transcript

Fig. 5 Immunogold electron microscope localization of ANS in mesocarp vascular tissue of grape berry. **a-b** Subcellular localization of ANS in sieve element. **b** The amplified portion of the boxed-in area of **a**, which shows that the ANS was mainly localized in the cytoplasm in sieve element. **c-d** Immunogold localization of ANS in companion cell. **d** Magnified image of the boxed-in area of **c**. The gold particles are mainly distributed in the cytoplasm and some also were found in the nucleus. **e-f** Negative controls of immunolocalization of ANS. **e** Antibody omission control; **f** preimmune serum control. No gold particles were detected. *SE* sieve element, *Cyt* cytoplasm, *CW* cell wall, *V* vacuole, *CC* companion cell, *N* nucleus. *Bars*=0.5 μ m



accumulation was observed in the leaves and stem phloem (Fig. 3). In grapevine leaves, ANS was mainly involved in the PAs biosynthesis; in stem phloem, ANS responded to both anthocyanins and PAs synthesis (Table 1). In leaf buds and petiole, the accumulation of ANS protein did not correspond to the levels of mRNA. A possible post-transcriptional regulatory mechanism may be involved, whereas in the stem xylem, a great quantity of ANS was investigated, but little PAs and anthocyanins were detected. Several authors have demonstrated the existence of a transport mechanism for several polyphenolic compounds, including flavonoids in grapevine (Fischer 1968; Berhow

and Vandercook 1989). The flavonoids synthesized in stem xylem might be transported to other tissues such as the root and stem tip, where no ANS protein was detected, but numerous of PAs and anthocyanins were accumulated (Table 1). The accumulation of flavonoids in different grapevine tissues is the result of in situ biosynthesis and a translocation process from the synthesis sites such as the fruit and leaves to the target organs (stem tip, root, and so on). However, the mechanism of flavonoid transport between different grapevine tissues needs to be made clear further.

Western blotting analyses have revealed ANS protein mainly in the fruit, leaves, stems, and leaf buds (Fig. 3). To

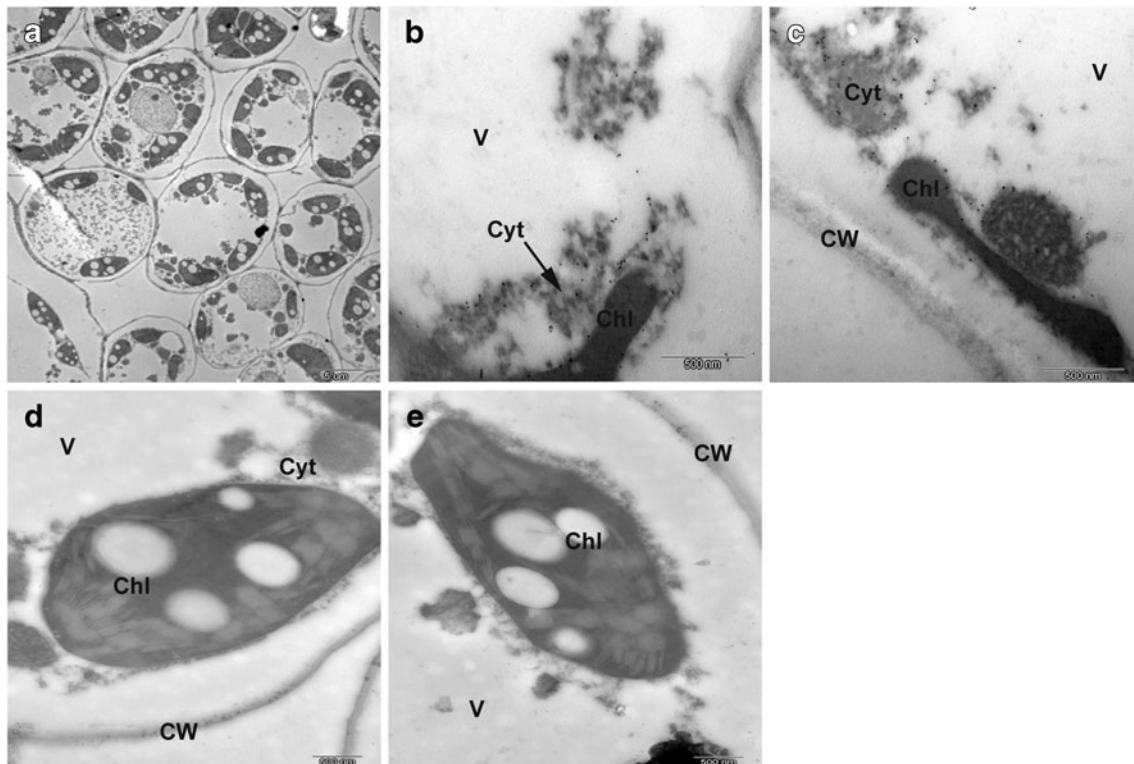


Fig. 6 Immunogold electron microscope localization of ANS in the leaves of young grape plants. **a** Ultrastructure of grapevine leaf cells; **b–c** ANS visualized by gold particles mainly resides in the cytoplasm ($\times 50,000$); **d–e** negative controls of immunogold electron microscopy localization of ANS in grapevine leaf cells. **d** Without the primary

ANS antibody; **e** the primary ANS antibody was substituted with the preimmune serum of rabbit. No gold particle was observed in these control cells. *CW* cell wall, *Cyt* cytoplasm, *V* vacuole, *Chl* chloroplast. *Bar*=10 μm in **a**, 0.5 μm in **b–e**

determine the exact localization of the ANS protein in these organs, an immunohistochemical analysis of fixed samples was performed with the anti-ANS IgG, which has high specificity and sensitivity (Wang et al. 2010). In grape berries, ANS was found to be distributed ubiquitously. The distribution of ANS in the exocarp and mesocarp has been discussed in our previous study (Wang et al. 2010). Here, we focused on the location of ANS in seed. Previous studies have suggested that flavonoids are localized not only in surface tissues of the seed coat, but also in the inner layers (Adams 2006; Pinelo et al. 2006; Cadot et al. 2006). Our present result showed that ANS was localized in the epidermis, outer integument, inner integument, and the cells around the raphe, which corresponded well to the distribution of PAs in grape seed (Cadot et al. 2006). It indicates that PAs accumulated in the different structures of grape seed are biosynthesized in situ. For vegetative organs, tissue-specific distribution of ANS in different organs was observed. Previous studies have detected that flavonoid biosynthetic enzymes are specifically localized in the epidermal cells in *Arabidopsis* and *Perilla* (Saito and Yamazaki 2002; Saslowsky et al. 2005; Saslowsky and Winkel-Shirley 2001). However, we found different dis-

tributed patterns of ANS in grapevine leaves and stems (Fig. 4d, g). These discrepancies imply that there exist different mechanisms of flavonoid biosynthesis and accumulation in different species (Braidot et al. 2008a). Although the physiological functions of anthocyanins and PAs biosynthesized in these tissues are not known, we cannot exclude the proposal that the sites of accumulation are not necessarily the sites of biosynthesis and that the transport of flavonoid end products between cells is involved (Hrazdina and Jensen 1992; Kaltenbach et al. 1999; Braidot et al. 2008a). The anthocyanins and PAs are transported to the epidermis to play an important role in protecting the tissues from unavoidable damages in grapevine. This is the first time to investigate the distribution of the ANS enzyme in leaf buds, but the roles the enzyme plays in these tissues need to be made clear. Meanwhile, there was little ANS signal observed in the root, which corresponded well to the result of Western blotting analysis.

Interestingly, the ANS signals were obviously distributed in vascular bundles (Fig. 4). Flavonoid 3'5'-hydroxylase has been only detected in the phloem in cotyledons and flower buds from *Catharanthus roseus* (Kaltenbach et al. 1999). It

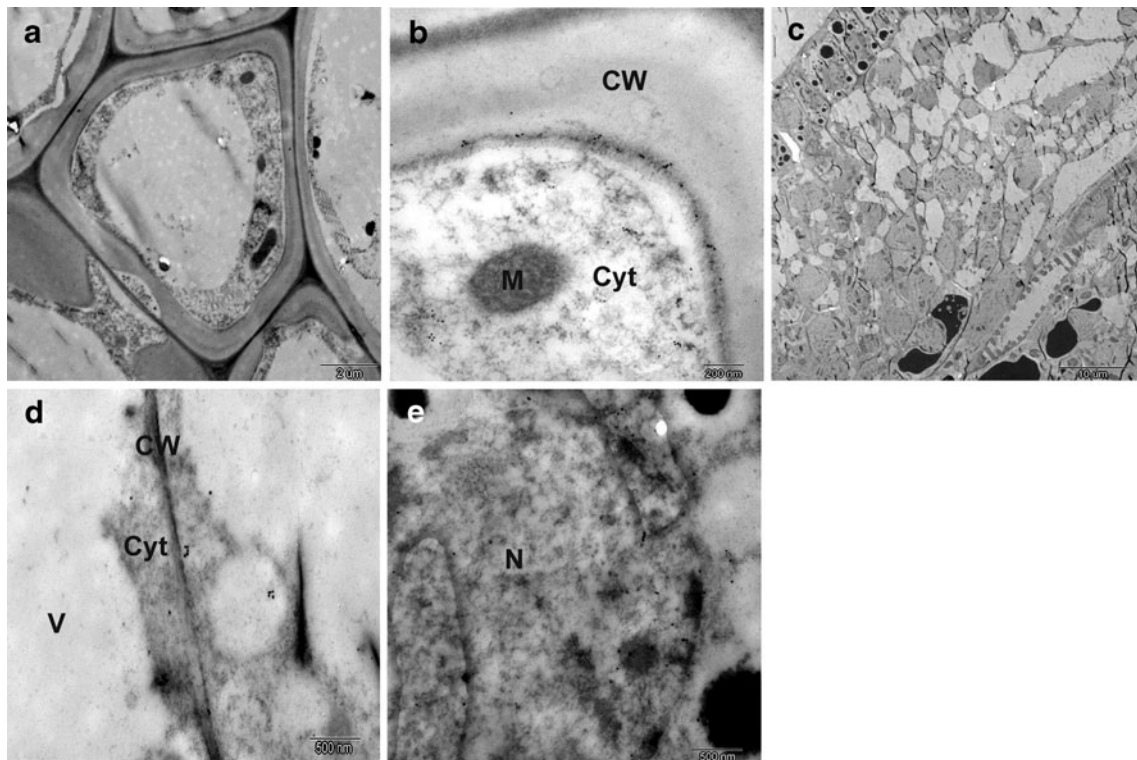


Fig. 7 Immunogold electron microscope localization of ANS in the stem (**a–b**), and leaf bud (**c–e**) of young grape plants. **a** Ultrastructure of grapevine stem cells; **b** ANS visualized by gold particles mainly resides in the cytoplasm ($\times 60,000$). Bar = 2 μm in **a**, 0.2 μm in **b**;

c ultrastructure of grapevine leaf bud cells; **c–e** gold particles representing ANS mainly resides in the cytoplasm (**d**) and the nuclear (**e**). Bar = 10 μm in **c**, 0.5 μm in **d–e**; CW cell wall, Cyt cytoplasm, V vacuole, Chl chloroplast, N nuclear

is suggested that there are some other flavonoid pathway enzymes distributed in the vascular bundles of grapevine, which combine with ANS to biosynthesize different flavonoid end products in situ. Many studies have confirmed the distribution of flavonoids in transport tissues (Gould et al. 2000; Gholami 2004). In addition, the vascular bundle was considered to function in the long distance transport of water, signaling molecules, and for nutrient reallocation (Oparka and Turgeon 1999; Bondada et al. 2005), and it is suggested that the flavonoids are transported from the vascular bundle to the different tissues/organs in grape plants subjected to biotic and abiotic stresses. Recent findings have confirmed flavonoid occurrence in vascular bundles, where they move towards the root tip (Buer et al. 2007). The identification of the flavonoid translocator BTL homolog associated to vascular bundles further indicated the existence of flavonoid transport in grapevine (Braidot et al. 2008b).

At the subcellular level, distributed patterns of ANS in the cytoplasm/plastid/nucleus have been found in the exocarp of developing grape berries in our previous study (Wang et al. 2010). However, in mesocarp vascular tissues, ANS was mainly localized in the cytoplasm, and the

nuclear distributed pattern of ANS was only observed in the companion cells (Fig. 5). The data show that ANS exhibits differences in subcellular localization between fruit skin and flesh, but its functions in different sites of grape berry need further study. Substantial studies have confirmed the cytoplasm ER-localized pattern of flavonoid pathway in vegetative tissues and suggested that flavonoid metabolism is catalyzed, not by free-floating “soluble” enzymes, but via one or more membrane-associated multienzyme complexes (Hrazdina and Jensen 1992; Jorgensen et al. 2005; Saslowsky and Winkel-Shirley 2001; Winkel-Shirley 1999). Here in the sampled grapevine vegetative tissues, the ANS enzyme was also mainly localized in the cytoplasm. It appeared that ANS was with other flavonoid enzymes organized into a complex to facilitate the synthesis of anthocyanins and PAs in the cytoplasm of different grapevine tissues. Pang et al. (2007) have found that *Medicago* ANS was localized in the cytosol. But the experiment was performed by particle bombardment of single-enzyme construct into tobacco leaf epidermal cells, not into the natural milieu of the endothelial layer of the *Medicago* seed coat. It cannot rule out possible complex formation when all the enzymes are present together in the

same cell. The nuclear distributed pattern of ANS was also detected in grape berry vascular bundle cells and leaf bud cells. It was not surprising. Several other recent reports have described the localization of some flavonoid enzymes in the nucleus and suggested that these specially localized enzymes are responsible for in situ synthesis of flavonoids in the nucleus (Chen et al. 2006; Naoumkina et al. 2007; Saslowsky et al. 2005; Tian et al. 2008; Yu et al. 2008). Much evidence has described the accumulation of flavonoids in the nucleus, which serves to protect DNA from UV and oxidative damage (Feucht et al. 2004; Polster et al. 2006; Saslowsky et al. 2005) and so on.

In conclusion, ANS was mainly distributed in grape berries, leaves, stems, petioles, and leaf buds in Cabernet Sauvignon grapevine. The ANS exhibits tissue-specific localization in different organs. At the subcellular level, the cytoplasm and nucleus distributed patterns of ANS were observed in different grapevine tissue cells. No matter what mechanisms were involved, the elucidation of the compartmentations of ANS in different grapevine tissues was of particular interest in the future.

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Conflict of interest The authors declare that they have no conflict of interest.

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