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Preparation and biological application of antibodies against leucoanthocyanidin reductase and anthocyanidin reductase from grape berry

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Summary

Proanthocyanidins (PAs) endow wine with the flavor of bitterness and astringency. Both leucoanthocyanidin reductase (LAR) and anthocyanidin reductase (ANR) are two key enzymes of PA biosynthesis in grape berries, but the previous studies on these enzymes only focused on the transcriptional expression of these genes. Here, the full-length cDNAs of *VvLAR1*, *VvLAR2* and *VvANR*, respectively, were cloned from wine grape berries and were then introduced into the pGEX-4T-1 expression vectors, which were highly expressed in Escherichia coli DH5α cells with the induction of the isopropyl-β-D-thiogalactoside (IPTG). The purified fusion proteins were used as the antigens to immunize rabbits, separately. The obtained antiserums were further purified to obtain the immunoglobulin G (Ig G) fractions, which were demonstrated to be capable of specifically immuno-recognizing the VvLAR1, VvLAR2 and VvANR from the crude protein extracts from grape berries with weight masses of approximately 43 kD. The analyses of translational expression of these enzyme genes during berry development and immunohistochemical localization of these proteins, by using the obtained antibodies, showed that a high amount of VvLAR1, VvLAR2 or VvANR was present at the pre-veraison stage and these enzyme proteins were all localized on the outer layer of the berry skin and the vascular bundle, as well as in the inner layer of the seed coat. This work provides an important basis for further studies on PA biosynthesis in grape berries.

Key words: LAR, ANR, polyclonal antibody, translational expression, immunohistochemical localization, grape berry.

Introduction

Proanthocyanidins (PAs), also known as condensed tannins, are oligomers or polymers of flavan-3-ol units and are polyphenolic secondary metabolites synthesized via the flavonoid biosynthetic pathway (Ferreira and Slade 2002, Marles *et al*. 2003). As one of the most ubiquitous groups of all plant phenolics, they are widespread throughout the plant kingdom with diverse biological and biochemical activities including protection against predation and pathogen attack (Lamb *et al*. 1989). In recent years, more and more attention has been drawn to PAs and their monomers because of their beneficial effects on human health, such as immunomodulatory and anticancer activities, antioxidant and radical scavenging function, anti-inflammatory activities, and antithrombotic effects (Rao *et al*. 2004, Sano *et al*. 2005, Subarnas and Wagner 2000, Zhao *et al*. 2007). Besides, PAs also provide the flavors of bitterness and astringency to beverages, especially the wines, where they have a significant influence on the mouth feel and the color alternation of the products (Lee *et al*. 2008, Peleg *et al*. 1999).

Leucoanthocyanidin reductase (LAR, EC 1.17.1.3) and anthocyanidin reductase (ANR, EC 1.3.1.77) are both key enzymes of the branch pathway of PA biosynthesis and are two members of the isoflavone reductase-like (IFR-like) group of the plant reductase-epimerase-dehydrogenase (RED) supergene family. They catalyze the NADPH-dependent conversion of leucoanthocyanidins into 2*R*,3*S*-flavan-3-ols [especially (+)-catechin] and anthocyanidins into 2*R*,3*R*-flavan-3-ols [especially (-)-epicatechin], respectively (Tanner *et al*. 2003, Xie *et al*. 2003). Since the isolation of the gene for LAR from *Desmodium uncinatum* and the functional identification of *BANYULS* (the gene encoding ANR in *Arabidopsis thaliana*) had been regarded as the landmarks in the research field of PA biosynthesis (Marles *et al*. 2003), a great deal of efforts have been put into the molecular cloning of the genes encoding LAR and ANR from various plants and their transcriptional regulation mechanism (Almeida *et al*. 2007, Bogs *et al*. 2007, Ikegami *et al*. 2007, Paolocci *et al*. 2007). Especially in grape, there is only one single gene (*VvANR*) encoding ANR, whereas there are two genes (*VvLAR1* and *VvLAR2*) encoding LAR (Bogs *et al*. 2005, Pfeiffer *et al*. 2006). During grape berry development, the transcription factor VvMYBPA1 specifically regulates PA synthesis through controlling the transcriptional expression of the genes encoding VvLAR1 and VvANR (Bogs *et al*. 2007). However, it hitherto remains unclear about the expression of these enzymes at the translational level, as well as their tissue and subcellular immunolocalization (Dixon *et al*. 2005, Xie and Dixon 2005).

In this paper, we report on the molecular cloning and expression of grape (*Vitis vinifera* L. 'Cabernet Sauvignon') *VvLAR1*, *VvLAR2* and *VvANR* genes in *Escherichia coli*, the purification of the fusion proteins, and the preparation

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of antibodies. These obtained polyclonal antibodies which have high level specificity and sensitivity are also used in immuno-detecting the translational expression and immunohistochemical localization of these enzymes in grape berries, which will surely be helpful to further studies in this field.

Material and Methods

General: The pGEX-4T-1 vector plasmid, the Glutathione Sepharose 4B and the HiTrap rProtein A FF were purchased from Amersham Pharmacia Biotech (UK), while all restriction enzymes were purchased from Takara (Japan). The Reverse Transcription System Kit and T4 Ligase were purchased from Promega (USA). All of the reagents used for PCR amplification and nucleotide purification were purchased from Tiangen Biotech (China). All oligonucleotide primers were synthesized by Bioasia Biological Company (China), and all PCR products were confirmed by sequencing at the same company. All the other chemicals were purchased from Sigma (USA) unless noted specially.

Plant material: Grape berries (*Vitis vinifera* L. 'Cabernet Sauvignon') were sampled at 3, 5, 7, 9, 11, 13 and 15 weeks after full bloom from a vineyard in the suburbs of Beijing in the year of 2007. The freshly harvested berries were selected randomly to remove those of physical injuries or infection and those developed abnormally. After being washed with distilled water and brief drying, the seeds of these berries were removed and the remainders were immediately frozen in liquid nitrogen and stored at -80 °C until use. The fresh grape berries sampled at 3 weeks after bloom were immediately fixed in the solution containing 4 % (v/v) paraformaldehyde and 2.5 % (v/v) glutaraldehyde overnight at 4 °C, dehydrated with a graded ethanol series and embedded in paraffin. These paraffin pieces would be used for analysis of immunohistochemical localization.

Total RNA extraction and purification: The total RNA was isolated from grape berries by using the improved method of cetyl triethyl ammonium bromide (CTAB) according to the reference of WEN *et al*. (2005). The total RNA was treated with RNase-free DNase to remove grape genome DNA and then purified with the RNA Purification Kit. The quality of the total RNA was verified by demonstration of intact ribosomal bands following agarose gel electrophoresis in addition to the absorbance ratios (A260/280).

Cloning of the full-length VvLAR1, VvLAR2 and VvANR genes: The appropriate mRNA was reversely transcribed to cDNA by using Reverse Transcription System Kit on the basis of the protocol of the supplier. According to the grape mRNA sequences of *VvLAR1*, *VvLAR2* and *VvANR* (GenBank accession number AJ865336, AJ865334 and DQ129684, separately) in GenBank, and the sequence of the pGEX-4T-1 *Escherichia coli* Expression vector plasmid, we selected the suitable restriction sites, designed and synthesized three pairs of specific primers for amplifying these genes as follows: VvLAR1 (F) 5'-CGGGATCCATGACTGTTTCTCCGGTTCCTTCG-3' and VvLAR1 (R) 5'-ACGCGTCGACTCAAGCGCAGGTTGCAGTGAC-3', VvLAR2 (F) 5'-GGAATTCATGACTGTTTTGTCTGTGAGTACTCC-3' and VvLAR2 (R) 5'-ACGCGTCGACTCAGGCGCAGGTAGCAGTGA-3', VvANR (F) 5'-CGGGATCCATGGCCACCCAGCACCCCATC-3' and VvANR (R) 5'-ACGCGTCGACTCAATTCTGCAATAGCCCCTTGGCC-3'(both the start and stop codon were underlined, and the restriction sites were also marked). The PCR reaction system of a 50 µl total volume was composed of 2 µl cDNA as the template, 1 µl of each primer (20 µM), 25 µl 2 × Pfu PCR MasterMix and 21 µL ddH_2O according to the protocol of the supplier with slight modification. The PCR reaction was carried out under the following conditions: predenaturation at 94 °C for 10 min, denaturation at 94 °C for 30 s, primer annealing at the temperatures showed below for 30 s and extension at 72 °C for 1 min for 30 cycles, a final extension at 72 °C for 10 min, and then cooling to 4 °C in a Techne TC-312 Thermal Cycler (UK). The annealing temperatures were as follows: *VvLAR1* 61.5 °C, *VvLAR2* 60 °C and *VvANR* 58 °C. The PCR products were isolated by 1.0 % agarose gel electrophoresis, and then purified by using TIANgel Purification Kit according to the operating instructions.

Reconstruction of expression vector: The purified PCR products and the pGEX-4T-1 plasmids were double-digested with the two designed restriction endonucleases separately as follows: *Bam*H I and *Sal* I for *VvLAR1*, *Eco*R I and *Sal* I for *VvLAR2*, and *Bam*H I and *Sal* I for *VvANR*. After the full length *VvLAR1*, *VvLAR2* and *VvANR* genes were sub-cloned into the corresponding double-digested pGEX-4T-1 vectors, the reconstructed plasmids pGEX-*VvLAR1*, pGEX-*VvLAR2* and pGEX-*VvANR* were then introduced into *Escherichia coli* DH5α cells separately. The transformation was verified by colony PCR, while further verification was conducted by extraction of plasmids and double restriction endonucleases analysis. Finally, the *Escherichia coli* DH5α cells separately harboring pGEX-*VvLAR1*, pGEX-*VvLAR2* and pGEX-*VvANR* were all sequenced from both sides of the inserted fragments.

Expression and purification of the recombination proteins: Expression of the fusion proteins was performed according to the method described by SAMBROOK and RUSSELL (2001) with slight modification. A single colony of *Escherichia coli* strain DH5α cells harboring the reconstructed plasmids was induced and cultured overnight at 37 °C in 10 ml Luria-Bertani (LB) liquid medium containing ampicillin (100 µg·ml). 100 µl of the culture was transferred into another 10 ml volume of fresh medium and was incubated for about 3 h until the optical density (OD600) of the cultured cells reached 0.8. Isopropyl-β-D-thiogalactoside (IPTG) was then added to the medium to induce the protein expression. The resulting fractions were assessed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method described by LAEMMLI (1970). Besides, a DH5α strain harboring the pGEX-4T-1 empty vector was induced and analyzed as control. Purification procedures were performed referring to the method of SAMBROOK and RUSSELL (2001) and the manufacturer's instruction for Glutathione

Sepharose 4B. After affinity chromatography, the purified proteins were concentrated by ultrafiltration (cut-off 8-10 kD) (Millipore, USA).

Antibody production: The purified and concentrated fusion proteins VvLAR1, VvLAR2 and VvANR were separated by SDS-PAGE, respectively. The gel which contained the fusion protein was cut off, ground in liquid nitrogen, and resuspended in an equal volume of phosphate buffered saline (PBS). First, 500 µg of the purified fusion protein were injected (subcutaneous) into a New Zealand rabbit after being emulsified with Freund's complete adjuvant. Six booster injections (500 µg per injection) were given at a 10-day interval, and the antiserum was collected 10 days after the last injection. Purification of the rabbit immunoglobulin G (Ig G) fractions was carried out by using HiTrap rProtein A FF column chromatography. The purified Ig G fractions were then verified by SDS-PAGE.

Analysis of antibody sensitivity: The immuno-recognizing sensitivity analysis of anti-VvLAR1, anti-VvLAR2 and anti-VvANR antibodies, respectively, was evaluated by Western blot, protein dot blot and enzyme-linked immunosorbent assay (ELISA) according to the methods described by Pan *et al.* (2005 a) and Zhang *et al.* (2001) with some modification. As for protein dot blot, the quality of fusion protein per well was in the range of 0.1 ng ~50 ng, and the Ig G fractions were diluted with PBS buffer to 2000-fold. The antibody sensitivity was defined as the minimum quantity of antigen that could be visualized in color by naked eyes after staining. In ELISA analysis, the purified fusion protein was coated on immunoplates at 1 µg per well and the antibodies of different concentrations diluted from 500-fold to 256,000-fold were used to react with the antigen on the immunoplates. The goat antibody against a rabbit Ig G conjugated with peroxidase (Promega, USA) was chosen as the secondary antibody, and was diluted to 2000-fold. The absorbance at 490 nm/630 nm ($OD_{490/630}$) was read in the ELISA reader (Thermo Multiskan MK3, USA), and the antibody titer was defined as the dilution times of the antibody corresponding to the net absorbance (OD_{490}-OD_{630}) above 0.500.

Analysis of translational expression and immunohistochemical localization of VvLAR1, VvLAR2 and VvANR proteins: The crude proteins were extracted from the pericarp of grape berries (the seeds discarded) of different developmental stages according to the method described by Pan e*t al.* (2005 b). The extraction buffer consisted of 50 mM Tris-HCl (pH 8.9), 5 mM ascorbic acid, 5 mM EDTA, 1 mM PMSF, 14 mM β-mercaptoethanol and 0.2% (w/v) PVPP. The separation of the extracted proteins was performed by SDS-PAGE in a 12 % polyacrylamide gel. The identical amount of protein (10 µg) was loaded per lane. After electrophoresis, the proteins were electro-transferred to nitrocellulose (0.45 µm, Amersham LIFE SCIENCE) using a transfer apparatus (Bio-Rad) and then were tested with the anti-VvLAR1, anti-VvLAR2 and anti-VvANR polyclonal antibodies, respectively, by Western blot to show the translational expression pattern of these enzymes.

The immunohistochemical localization of VvLAR1, VvLAR2 and VvANR proteins in grape berries was carried out as described by Hou and Huang (2004). The paraffin pieces were sectioned into 8-µm slices. The secondary antibody, anti-rabbit Ig G-alkaline phosphatase-conjugate (Promega, USA) was diluted to 50-fold and the Western Blue stabilized substrate for alkaline phosphatase (Promega, USA) was modified to 100 µl per slice.

Results and Discussion

Cloning the full-length genes and construction of the expression vectors: Agarose gel electrophoresis analysis of the total RNA isolated from grape berries and the purified RNA showed that the obtained RNA had good purity and was qualified for further use. As shown in Fig. 1, strong bands of PCR products were clearly detected on 1.0 % agarose gel, and the fragments of *VvLAR1*, *VvLAR2* and *VvANR* were 1059 bp, 1106 bp and 1035 bp, respectively (including the open reading frame, restriction enzyme sites and protective bases). After the reconstructed plasmids pGEX-*VvLAR1*, pGEX-*VvLAR2* and pGEX-*VvANR* were separately transferred into the *Escherichia coli* DH5α cells, colony PCR and double restriction endonucleases analysis were used to confirm the success of the transformation. These reconstructed plasmids were sequenced from both sides and BLAST analysis indicated that the PCR products had 100 % identity in the activity region with the published genes of *VvLAR1*, *VvLAR2* and *VvANR*. The results suggested that the nucleotide sequences of them were correct.

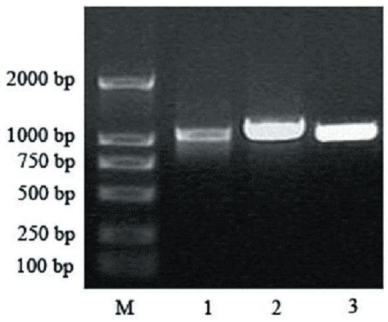

Fig. 1: Agarose gel electrophoretic analysis of PCR products of *VvLAR1*, *VvLAR2* and *VvANR* genes. M indicates DNA marker DL2000; Lane 1-3 indicate PCR products of *VvLAR1*, *VvLAR2* and *VvANR* genes, respectively.

Expression and Purification of the fusion proteins: When the transformed *Escherichia coli* DH5α cells were induced at 30 °C with the IPTG concentration of 0.4 mM for 3 h, the expression amount of the three kinds of fusion proteins all reached comparatively high levels. Crude lysates of the samples were taken and analyzed by SDS-PAGE, as shown in Fig. 2. The molecular masses of the glutathione S-transferase (GST) fusion proteins VvLAR1, VvLAR2 and VvANR were estimated approximately 65 kD according to the amino acid sequences, which was in good agreement with the sizes of the expressed proteins. Besides, no fusion protein was detected in the control of either *Escherichia coli* DH5α strains harboring the pGEX-4T-1 empty vector that was induced

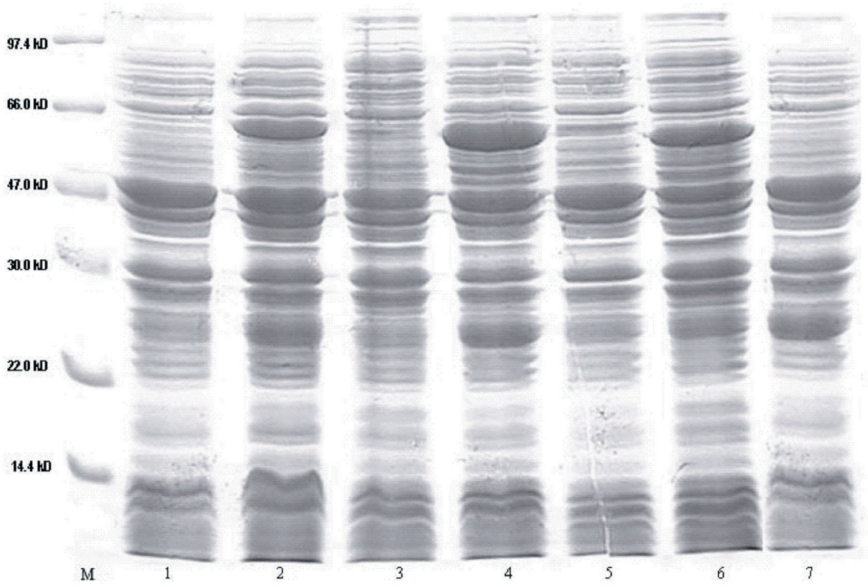

Fig. 2: Analysis of expression products by SDS-PAGE patterns of total cellular protein under inducing and non-inducing conditions by Coomassie Brilliant Blue R-250 staining. M indicates Low Rang Protein Molecular Weight Marker; Lane 1, 3 and 5, *E. coli* strain DH5α cells harboring pGEX-*VvLAR2*, pGEX-*VvLAR1* and pGEX-*VvANR* without induction; Lane 2, 4 and 6 *E. coli* strain DH5α cells harboring pGEX-*VvLAR2*, pGEX-*VvLAR1* and pGEX-*VvANR* with induction by IPTG; Lane 7, *E. coli* strain DH5α cells harboring bare vector pGEX-4T-1 with induction by IPTG.

with the same method or the transformed *Escherichia coli* DH5α cells that were not induced. The analysis of quantitative thin layer scanning showed that the expression of VvLAR1, VvLAR2 and VvANR fusion proteins was almost 40%~50 % of the total cellular protein.

The recombinant proteins containing GST·Tag sequence could be bound to the Glutathione Sepharose 4B to facilitate the purification process. However, only the soluble GST·Tag fusion proteins in their nature form could be bound and purified by the GST·Bind resin. Because most of the fusion VvLAR1, VvLAR2 and VvANR proteins seemed to be contained in inclusion bodies, it was necessary to purify, denature and then renature the inclusion bodies. In order to improve the efficiency of the chemical treatment, the harvested cells were first washed with PBS buffer to reduce the interference of the remnant medium, and the steps of washing the inclusion bodies were repeated to remove the contaminated proteins as completely as possible. As a result, the fusion proteins in the precipitates reached almost more than 90 % of the total protein and could be directly used as antigen, which was consistent with the descriptions of HARLOW *et al.* (1988). The fusion proteins purified by affinity chromatography were detected by SDS-PAGE, showing two main fractions, the molecular weights of which were consistent with those of the proteins fused with GST·Tag and the empty GST·Tag proteins (Fig. 3). The results indicated that the target genes had been correctly expressed into the target proteins.

Antibody specificity: The Ig G fractions against VvLAR1, VvLAR2 and VvANR, which were purified by using affinity column chromatography, were detected by SDS-PAGE. The results showed two bands, a heavy chain (50 kD) and a light chain (25 kD), indicating the integrality of the Ig G fractions. For analysis of Western blot, the recombinant proteins were used as the antigens

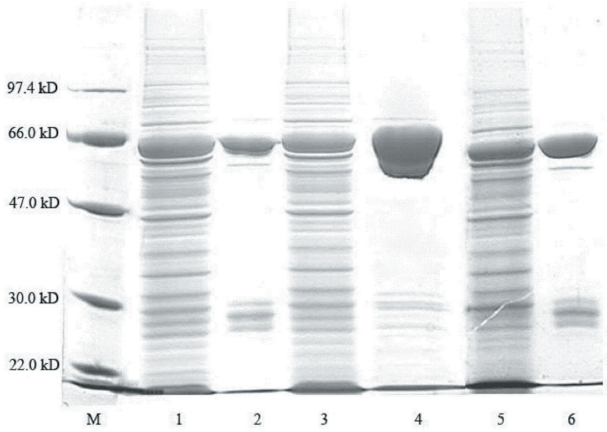

Fig. 3: Coomassie brilliant blue R-250 stained SDS polyacrylamide gels of the purified recombinant VvLAR1, VvLAR2 and VvANR proteins. M indicates Low Rang Protein Molecular Weight Marker; Lane 1, 3 and 5, the Inclusion Bodies Lysate obtained from *E. coli* strain DH5α cells harboring pGEX-*VvLAR1*, pGEX*VvLAR2* and pGEX-*VvANR,* separately; Lane 2, 4 and 6, the purified fusion proteins VvLAR1, VvLAR2 and VvANR.

and the results showed that all the three kinds of antibodies could immuno-recognize the antigens clearly at the level of 1 μg (Fig. 4). Further more, there was no cross reaction of the antibody for VvLAR1 with VvLAR2 protein and vice versa, as well as that of VvANR. Assays of protein dot blot showed that the anti-VvLAR1, anti-VvLAR2 and anti-VvANR antibodies could all specifically immuno-recognize VvLAR1, VvLAR2 and VvANR, but could not immuno-react with bovine serum albumin (BSA), which suggested that they had high degrees of detectable sensitivity. Both anti-VvLAR1 and anti-VvLAR2 antibodies diluted to 2000-fold could recognize 3.125 ng of antigen, while anti-VvANR antibody had a higher degree of detectable

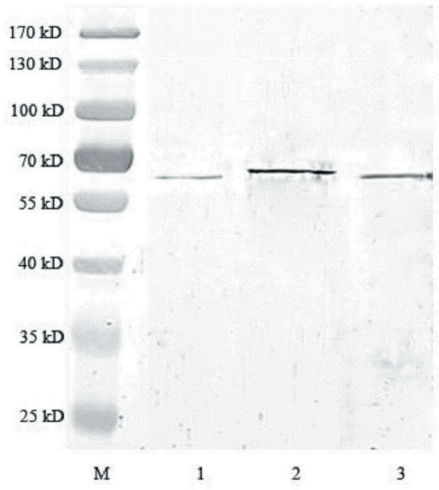

Fig. 4: Western blot analysis of the purified fusion proteins, VvLAR1, VvLAR2 and VvANR with their corresponding antibodies, respectively. M indicates Prestained Protein Ladder; Lane 1-3 indicate immunobloting of purified recombinant proteins VvLAR1, VvLAR2 and VvANR which fused with GST·Tag.

sensitivity, which could recognize 0.375 ng of antigen at the same dilution ratio of antibody. ELISA analysis also revealed high levels of immune sensitivity of these antibodies. For anti-VvLAR1 and anti-VvLAR2 antibodies, the net absorbance ($OD_{490\ nm}$-$OD_{630\ nm}$) of 1/64,000 dilution of the antibodies was above 0.5, nevertheless the net absorbance of control (1/2,000 dilution of the serum colleted before immunity) was still below 0.1, which indicated that the titers of polyclonal antibodies anti-VvLAR1 and anti-VvLAR2 were all above 64,000. In contrast, the titer of anti-VvANR antibody was much higher, even more than 128,000. Therefore, the obtained three kinds of polyclonal antibodies with high levels of immune specificity and sensitivity could be surely used in lots of further studies in this field, such as immumohistochemical and immunocellular localization of these enzymes, as well as protein-protein interactions by immuno-coprecipitation technique.

Western blot analysis of VvLAR1, VvLAR2 and VvANR proteins during the development of grape berries: In order to investigate the expression of *VvLAR1*, *VvLAR2* and *VvANR* genes at the translational level during the development of grape berry, the crude protein extracts from the berry pericarp of different developmental stages were separated by SDS-PAGE firstly, and then were detected with these polyclonal antibodies by Western blot, respectively. As shown in Fig. 5, a single peptide with molecular mass of approximately 43 kDa was specially detected from the protein fractions of the berry tissues, using antibody raised against grape berry VvLAR1, VvLAR2 or VvANR, indicating that the VvLAR1, VvLAR2 and VvANR in grape berries may be all composed of 43 kDa subunit(s). The mass weights of these enzyme proteins were also consistent with the descriptions of previous studies (Bogs *et al*. 2005), which proved the specificity of these antibodies used in this experiment. The mass weight of immuno-detected peptide did not change throughout berry development. However, the intensity of immunoblotting signal differented in VvLAR1, VvLAR2 or VvANR from berries of different developmental stages, on the basis of the same protein concentrations, with the same experimental procedures and using the antibody mentioned above. At the early stages of grape berry development (3-7 weeks after full bloom), the expression intensity of VvLAR1, VvLAR2 and VvANR at translational levels all reached their highest levels, which were associated with the accumulation of the precursors of PAs and free monomers. After that, the translational expression of VvLAR1, VvLAR2 and VvANR all decreased dramatically before véraison (10 weeks after full bloom), and then maintained at a low level until completely ripening. Such translational expression dynamics of VvLAR1 and VvANR, accompanying to berry development, was both consistent with the previous descriptions concerning their transcriptional expression changes, whereas that of VvLAR2 was partially different, which indicated that they might be regulated by different mechanism (Bogs *et al.* 2005, 2007).

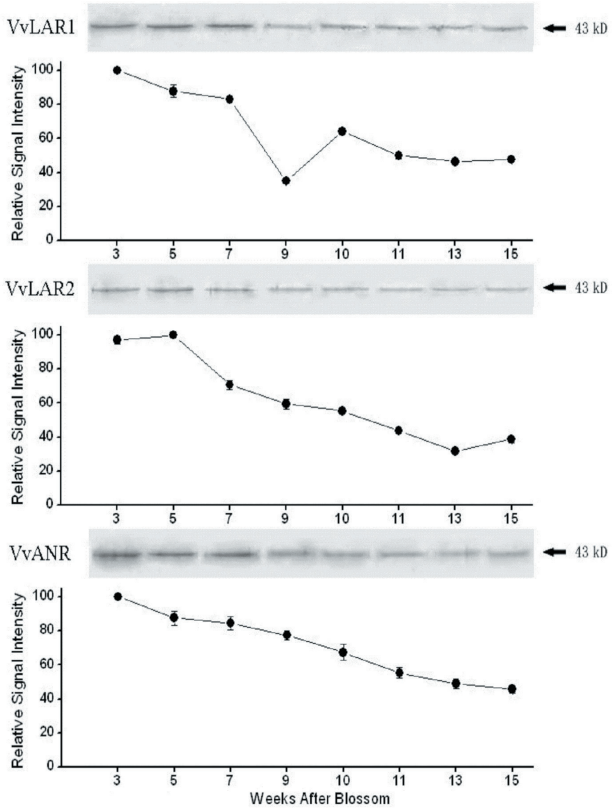

Fig. 5: Western blot analysis of VvLAR1, VvLAR2 and VvANR in the crude protein extracts of pericarp from different periods of berry development (3, 5, 7, 9, 10, 11, 13, 15 weeks after full bloom) and the analysis of their relative signal intensity. The signal intensity of each band is performed by Sigmascan software. The relative intensity of the band with the greatest immunosignal in a sheet of membrane of Western blotting is defined as 100. The data l are means ± standard error of three repetitions and these repetitions are from different protein extracts.

Immunohistochemical localization of VvLAR1, VvLAR2 and VvANR proteins in grape berries: The tissue localization of VvLAR1, VvLAR2 and VvANR proteins in grape berries was investigated by using immunohistochemistry technique

(Colored figures not shown). The grape berries of 3 weeks after full bloom were selected as the test samples, because of the higher expression intensity of these enzyme genes and the manipulability of the berry samples. A similar distribution among VvLAR1, VvLAR2 and VvANR proteins was present in the berry tissues. These enzyme proteins all largely localized on the outer layer of the berry skin and the vascular bundle, while slightly in the parenchyma cells. VvLAR1, VvLAR2 and VvANR were particularly abundant in the inner layer of the seed coat (inner integument), and were also present in the cells of the seed embryo at relatively lower levels. In the grape berries, anthocyanins were found almost exclusively in the skin cells and only slightly in the flesh cells (NUNEZ *et al.* 2004), while proanthocyanidins were also detected mostly in the skins and the seeds and only a little in the flesh cells (PRIEUR *et al.* 1994; SOUQUET *et al.* 1996). According to these reports, it is obvious that the berry tissues where VvLAR1, VvLAR2 and VvANR proteins are highly expressed are the right places where the free flavan-3-ol units and PAs are largely accumulated (BOGS *et al*. 2005).

In order to verify the reliability of immunolocalization technique, two controls were carried out, one omitting the primary antibody of VvLAR1, VvLAR2 or VvANR and another substituting the primary antibody with per-immune rabbit serum, while all the other procedures proceeded as usual. The results showed that almost no blue-signal representing VvLAR1, VvLAR2 or VvANR protein was detected in these sections, which indicated that the immunohistochemical localization technique used in the study was reliable. Meanwhile, it suggested that the antibody used in this study was highly specific and further demonstrated that the background color was very light.

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