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Screening and verification of late embryogenesis abundant protein interacting with anthocyanidin reductase in grape berries

Lin Mu[1)], Fei He[1)], Qiu-Hong Pan, Li Zhou and Chang-Qing Duan

Center for Viticulture and Enology, College of Food Science and Nutritional Engineering, China Agricultural University, Beijing, China

Summary

Anthocyanidin reductase (ANR, EC 1.3.1.77) catalyzes the conversion of anthocyanidins to 2R, 3R-flavan-3-ols in *Vitis vinifera* grapes. These are basic structural units of proanthocyanidins (PAs). The regulation of PA biosynthesis at protein level is not yet clear. Here, we find a VvANR interaction partner called late embryogenesis abundant protein (LEA), which can interact with VvANR in the yeast two-hybrid (Y2H) system. We verified the interaction between VvANR and VvLEA by Y2H and co-immunoprecipitation (CoIP) in yeast *in vitro*, and using the firefly luciferase complementation imaging (LCI) system *in vivo*. Additionally, the co-localization of VvANR and VvLEA in *Arabidopsis thaliana* protoplasts also provids the essential conditions for their interaction. The different expressions of VvANR and VvLEA depended both on the age of the grape berries and on the duration of cold treatment. These findings provide primary evidence for protein-regulation and the potential formation of multi-enzyme complex of VvANR, as well as of the PA biosynthesis.

Key words: anthocyanidin reductase, late embryogenesis abundant protein, *Vitis vinifera*, yeast two-hybrid, protein-protein interaction.

Introduction

Proanthocyanidins (PAs) (also referred to as condensed tannins) are oligomers or polymers of flavan-3-ol units, synthesized via the flavonoid metabolism pathway. PAs can protect plants from bacterial and fungal infection, from the attentions of herbivorous animals and insects, and can even inhibit the growth of nearby plants (Bais *et al.* 2003, Koes *et al.* 2005). PAs and their monomers contained in the human diet also have beneficial effects on health including: antithrombotic effects, immunomodulatory and anticancer effects and anti-inflammatory effects (Rao *et al.* 2004, Sano *et al.* 2005, Zhao *et al.* 2007). The majority of the PAs found in red wine are grape-derived. PAs participate in the mouth's tactile perception and the 'feel' of a red wine because of their astringent, bitter properties. They also play important roles in the long-term color stability of red wines (Kennedy 2008, Chira *et al*. 2009, Jordão *et al*. 2010, Gris *et al*. 2011, Jordão *et al*. 2012). Therefore, studies on the biosynthesis and regulation of PAs in *Vitis vinifera* have attracted much attention due to their important contribution to quality in grapes and in wines. However, previous research in this field was mainly focused on the transcriptional regulation of the structure genes encoding the key enzymes, such as the *Myc* family, the *Myb* family, WD40 protein, WRKY and TFIIIA protein 'WIP'. (Marles *et al*. 2003, Deluc *et al*. 2006, 2008, Bogs *et al*. 2007, Czemmel *et al*. 2009, 2012, Terrier *et al*. 2009; Azuma *et al*. 2012). It remains unclear today, whether PA biosynthesis is regulated at the protein level, so it is worthwhile identifying any potential 'protein-protein regulatory factors' or the multi-enzyme complex members (Winkel-Shirley 1999, Jørgensen *et al.* 2005).

Anthocyanidin reductase (ANR) is a key enzyme in PA biosynthesis, which has been reported to catalyze the conversion of anthocyanidins to 2*R*, 3*R*-flavan-3-ols such as (-)-epicatechin, (-)-epigallocatechin and (-)-epicatechin-3-*O*-gallate (Bogs *et al*. 2005). To identify a possible regulation mechanism for PA biosynthesis at the protein level, yeast two-hybrid (Y2H) was used to screen and test proteins which might interact with the ANR in *Vitis vinifera* (VvANR). One gene of the late embryogenesis abundant protein (LEA) family was discovered from these positive VvANR interaction partners, and the interaction between VvLEA and VvANR was then confirmed both *in vitro* and *in vivo*. LEA, which was first reported in the cotyledons of cotton, *Gossypium hirsutum*, is usually more abundant in late embryogenesis than in mid embryogenesis or in the early germination period (Dure III *et al*. 1981, Galau *et al*. 1986). However, it has been reported that LEA can exist in many plant organs and its expression can be induced by a large number of factors including: drought, low temperature, salt damage and abscisic acid (ABA). Environmental stresses are important factors regulating plant secondary metabolism and LEA could help plants to avoid injury during exposure to these (Park *et al*. 2003, Zegzouti *et al*. 1997). Findings in this area are likely to provide important evidence for discerning the regulation of VvANR catalysis at protein level, as well as of PA biosynthesis, and the potential multi-enzyme complex member works with VvANR.

Material and Methods

Plant materials and growth conditions: *Arabidopsis thaliana* plants (ecotype Columbia-0) were used in this study for the analysis of subcellular co-lo-

Correspondence to: Dr. Chang-Qing Duan, Center for Viticulture and Enology, College of Food Science and Nutritional Engineering, China Agricultural University, 17, Tsinghua East Road, 100083 Beijing, China. Fax: +86-10-62737136. E-mail: chqduan@cau.edu.cn

calization of VvANR and VvLEA. *Nicotiana benthamiana* plants were used to verify the interaction between VvANR and VvLEA. These plants were grown in potting mix in a growth chamber, with illumination of about 120 μmol photons $m^{-2}s^{-1}$ over a 16 h photoperiod. Ten-year-old *Vitis vinifera* L. cv. Cabernet Sauvignon grapevines were grown in commercial vineyards in Beijing, China. Grape berries were sampled in 2011 at four weeks after full bloom (AFB) (before véraison), at eight weeks AFB (during véraison) and at 12 weeks AFB (after véraison). Representative samples of the whole vineyard population were taken according to the method of BOULTON *et al.* (1995). On each sampling date, three 100-berry samples were collected from seven 10-cluster selections at similar positions on at least 50 different vines. Sampling time was always between 10 am and 11 am. Berry samples were carried back to the laboratory within two hours. After washing in distilled water, samples were dried with a clean cotton cloth at room temperature before cold treatment.

The screening of the VvANR interaction partner by Y2H and the cloning of its Open Reading Frame (ORF): Bait cloning and Y2H screening to obtain the interacting proteins were carried out following the manufacturer's instructions (Clontech). The coding sequences of *VvANR* were cloned into pGBKT7 (Clontech) as baits and transformed into AH109 (Clontech) yeast strain. The cDNA library was constructed using total RNA isolated from 'Cabernet Sauvigno' berries at eight weeks AFB, and transformed into AH109 yeast strain for yeast mating.

After a sequence comparison in the National Center for Biotechnology Information (NCBI) database with BLAST, the VvANR interaction partner was found to be part of the C-terminal sequence of VvLEA (C-VvLEA). Thus the whole ORF of the VvANR interaction partner (called *VvLEA*) was amplified by PCR using the forward primer 5'-ATGGCTGATCGAGTTCACCC-3', and the reverse primer 5'-TTACCAAAGTCTCACGCTGA-3'.

Analysis of protein interaction by Y2H and co-immunoprecipitation (CoIP) in yeast: The putative positive clones from Y2H screening were confirmed by Y2H test following the manufacturer's instructions (Clontech).

CoIP tests were performed using extracts of yeast cells (SHANG *et al*. 2010). Yeast strains were grown on SD medium deficient in Leu, Trp, His, and Ade (SD-4) to OD_{600} 1.0 at 30 °C. The total protein samples of positive clones were prepared with an extraction buffer (2 mL·g^{-1} cells) containing 50 mM HEPES (pH 7.4), 10 mM EDTA, 0.1 % (v/v) Triton X-100, 1 mM PMSF, and 1 μg·mL each of aprotinin, leupeptin, and pepstatin A (Sigma-Aldrich). The antiserums used in CoIP were the antiserums (Bio-Med Biotechnology) specific against MYC-tagged protein (in pGBKT7) and HA-tagged protein (in pGADT7), respectively. The immunoprecipitation experiments were performed with protein A/G Plus-agarose beads (Santa Cruz Biotechnology) following the manufacturer's protocol. First, the cell lysates, the protein A/G Plus-agarose beads and the antiserum against HA-tagged protein were incubated together at 4 °C overnight in the extraction buffer. Second, the beads were thoroughly washed twice with buffer A (50 mM Tris, pH 8.0, and 150 mM NaCl, and 0.1 % [v/v] Triton X-100) and buffer B (50 mM Tris, pH 8.0, and 0.1 % [v/v] Triton X-100) and resuspended with SDS-PAGE sample buffer. The CoIP were separated on a 10 % SDS-PAGE and analyzed by Western blotting with anti-MYC serum (PAN *et al*. 2005). First, the proteins on the 10 % polyacrylamide gels were electrophoretically transferred to nitrocellulose membranes (0.45 μm, Amersham Biosciences, Little Chalfon, Buckinghamshire, UK) in 25 mM Tris-HCl (pH 8.3), 192 mM glycine and 20 % (v/v) methanol. Second, the blotted membranes were pre-incubated for 2 h in a blocking buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 3% [w/v] BSA, 0.05 % [v/v] Tween-20), then incubated with gentle shaking for 3 h at room temperature in the antiserum against MYC-tagged protein (diluted 1:1000 in the blocking buffer). Third, after being extensively washed with TBST1 (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05 % [v/v] Tween-20), the membranes were incubated with goat anti-mouse IgG which had been conjugated with alkaline phosphatase (diluted 1:1000 in the TBST1) at room temperature for 1 h, and then washed with TBST2 (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% [v/v] Tween-20) and TBS (10 mM Tris-HCl, pH 7.5, 150 mM NaCl). The proteins detected were visualized by inoculating the membranes in BCIP/NBT (Promega).

Transient expression in *A. thaliana* protoplasts: To observe the subcellular co-localization of VvANR and VvLEA, the corresponding cDNA of the ORFs of *VvANR* and *VvLEA* were amplified with the forward primers 5'-CCGCTCGAGATGGCCACCCAGCACCCCAT-3', 5'-CCTTAATTAAATGGCTGATCGAGTTCACCC-3' and the reverse primers 5'-CGGGATCCCGATTCTGCAATAGCCCCTTG-3', 5'-AGGCGCGCCACCAAAGTCTCACGCTGAAAT-3', respectively. The transcription of *VvANR* was driven by the *Cauliflower mosaic virus* (CaMV) 35S promoter and VvANR was downstream-tagged by green fluorescent protein (GFP). The 35S promoter-driver and GFP-cDNA were ligated into the pMD 19-T vector (Takara) at the *Pst*I (5'-end) and *Eco*RI (3'-end) sites, forming the vector called GFP-vector (ZHAO *et al.* 2011a). The cDNA of *VvLEA* was cloned to the *Pac*I (5'-end) and *Asc*I (3'-end) sites of the pMD 19-T vector harboring mCherry (a red fluorescent protein, RFP) (ZHAO *et al.* 2011a).

According to Sheen's protocol (http://genetics.mgh.harvard.edu/sheenweb/), protoplasts were isolated from leaves of three- to four-week old *A. thaliana* plants (ecotype Columbia-0), and were co-transformed by the VvANR-GFP and VvLEA-mCherry construct pair, to observe whether VvANR was co-localized with VvLEA. The fluorescence of GFP and mCherry was observed using a confocal laser scanning microscope (Nikon C1, Nikon Corporation, Tokyo, Japan) after incubation at 23 °C for 16 h. The mCherry fluorescence and GFP fluorescence were excited respectively with a 543 nm HeNe green laser and 488 nm argon-ion laser, filtered with a 545 nm spectroscope. The mCherry fluorescence was detected with a

585-615 nm filter set and the GFP fluorescence was detected with a 505-530 nm filter set.

Test of protein-protein interaction by firefly luciferase complementation imaging (LCI): A luciferase (Luc) complementation imaging (LCI) assay was used according to procedures described previously (Chen *et al.* 2008), where the firefly Luc enzyme is divided into the N-terminal (NLuc) and C-terminal (CLuc) halves, which do not reassemble or function spontaneously. When the two fused proteins interact, Luc enzyme can be reconstituted and the Luc activity can be observed. The primers for cloning VvANR-NLuc were: the forward primer 5'-GGGGTACCATGGCCACCCAGCACCCCAT-3' and the reverse primer 5'-CGGGATCCGGATTCTGCAATAGCCCCTTGG-3'; for VvANR-CLuc these were: the forward primer 5'-GGGGTACCATGGCCACCCAGCACCCCAT-3' and the reverse primer 5'-CGGGATCCTCAATTCTGCAATAGCCCCT-3'; for VvLEA-NLuc these were: the forward primer 5'-GGGGTACCATGGCTGATCGAGTTCACCC-3' and the reverse primer 5'-CGGGATCCGGCCAAAGTCTCACGCTGAAAT-3'; for VvLEA-CLuc these were: the forward primer 5'-GGGGTACCATGGCTGATCGAGTTCACCC-3' and the reverse primer 5'-CGGGATCCTTACCAAAGTCTCACGCTGA-3'. The cDNAs were all cloned to the *Kpn*I (5'-end) and *Bam*HI (3'-end) sites of the NLuc and CLuc vectors, respectively. *Agrobacterium tumefaciens* (GV3101) was transformed with these constructs, and the bacterial suspensions with the same concentration were infiltrated into young but fully-expanded leaves of seven-week old *N. benthamiana* plants using a syringe without a needle. After infiltration, the plants were kept in darkness for 12 h and afterwards in 16/8 h light/dark cycles for 60 h at room temperature. Luc activity was detected with a CCD imaging camera (iXon DV887DCS-BV, Andor Technology, Belfast, UK).

Cold treatment of 'Cabernet Sauvignon' berries: On each sampling day, 'Cabernet Sauvignon' berries were evenly divided into eight groups (180-200 berries per group). Four groups were incubated at 5 °C and four at 25 °C (control) in the dark (relative humidity 70 %). After incubation for 4, 12, 20 or 28 h, one group at 5 °C and one at 25 °C were frozen in liquid nitrogen and stored at -80 °C.

Real-time PCR: The total RNA of grape berries was extracted using a cetyltriethylammnonium bromide (CTAB) extraction buffer (He *et al.* 2009). All RNA samples were incubated with DNase I (RNase-free, TaKaRa, Japan) and purified using an EZ-10 Spin Column RNA Purification Kit (BioBasic, Canada). The quality of RNA was verified by checking intact ribosomal bands by agarose gel electrophoresis and the absorbance ratios (A260/A280). All purified RNA samples were adjusted to the same concentration of 50 ng/µl with Rnase-free water, and reverse-transcribed into cDNA using AMV reverse transcriptase (Takara, Japan) following the manufacturer's instructions.

Quantification of the transcripts was done using SYBR® *Premix Ex Taq*™ (TaKaRa, Japan) on a 7300 Real Time PCR System (Applied Biosystems, USA). Each real-time PCR reaction (20 µL) contained 2.0 µl of the cDNA template (100 ng/µl), 1.6 µL of the primer mixture (10 µM each, mixed with equivalent volume), 10.0 µl of 2 × SYBR *Premix Ex Taq* II and 0.4 µL of 50× ROX Reference Dye (Takara, Japan), and 6.0 µL ddH_2O. The template cDNA was denatured at 95 °C for 30 s followed by 40 cycles of amplification with 95 °C for 10 s, 60°C for 31 s and a melt cycle from 60 °C to 95 °C. *VvANR*, *VvLEA* and *VvUbiquitin* were analyzed simultaneously. Each sample was processed in two replicates to obtain two pieces of RNA, then reverse-transcribed into cDNA, and each cDNA sample was processed in three technical replicates of Real-time PCR.

The primers of *VvANR* and *VvUbiquitin* for real-time PCR were referred to previous report (Bogs *et al.* 2005), with forward 5'-CAATACCAGTGTTCCTGAGC-3', and reverse 5'-AAACTGAACCCCTCTTTCAC-3', forward 5'-GTGGTATTATTGAGCCATCCTT-3' and reverse 5'-AACCTCCAATCCAGTCATCTAC-3', respectively. The primers specific for *VvLEA* were forward 5'-ATGTCACCGTGTTCCAGACTGC-3' and reverse 5'-TCACCAAGGTTTCCTTCATGCC-3' designed using the PRIMER PREMIER 5.0 program (PREMIER Biosoft International, California, USA). The efficiency of these primers was tested in preliminary experiments with templates of dilutions of the purified PCR product and maintained a γ^2 value $\geq$ 0.98. *VvUbiquitin* cDNA was used as an internal control. The specificity of primers was verified on the basis of the following experiments. First, using cDNA from grape berries as a template, specific fragments were obtained through PCR amplification. Fragment sizes were 182 bp for *VvUbiquitin*, 141 bp for *VvANR*, and 75 bp for *VvLEA*. These fragments were sequenced from both the 3′-end and the 5′-end, and compared with nucleotide sequences published in Genbank. The results confirmed that they were identical. Second, the dissociation curve of each gene indicated the absence of primer dimer or other non-specific products. The dissociation curve was obtained under conditions of 95 °C for 15 s and 60 °C for 1 min.

The relative transcript abundance for each gene was quantified by normalizing to the expression level of *VvUbiquitin*. Every run, for each sample, included the *VvUbiquitin* control. The $2^{-\Delta Ct}$ ($\Delta Ct = Ct_{Target} - Ct_{Ubiquitin}$, Ct: cycle threshold) was used to obtain the normalized expression level of target genes (Bogs *et al.* 2005).

Results and Discussion

Screening for interaction partners of VvANR in Y2H from which the ORF (804 bp) was cloned and sequenced: In the Y2H screen using VvANR as bait, a VvANR interaction partner was found which was a part of the C-terminal sequence (amino acid 129 to 267) of VvLEA (C-VvLEA). The ORF of *VvLEA* was then cloned which was 804 bp. The Pfam Database of Protein families categorizes six LEA groups (Hundertmark and Hincha 2008, Punta *et al.*

2012). VvLEA, found in this study, belonged to group 2 (LEA_2). The nucleotide sequence of the *VvLEA* ORF we cloned from 'Cabernet Sauvignon' differed in only one base compared with the reference cDNA-sequence in the NCBI database for *V. vinifera* 'Pinot Noir', while the amino acid sequences were identical. Nucleic acid sequence alignments of *VvLEA* with *LEA*-genes of other dicotyledonous plants (including *Prunus persica*, *Populus trichocarpa* and *Medicago truncatula*) resulted in 53-63% identity.

Validation of protein interaction by Y2H and CoIP in Yeast: The interaction of the VvANR interaction partner and VvANR, the ORF of VvLEA and VvANR were confirmed with Y2H and CoIP assays in yeast, respectively. Both the cDNA-sequence of VvANR interaction partner and *VvLEA* were cloned to pGADT7, transformed into AH109 yeast strain with VvANR/pGBKT7, respectively. For negative controls, C-VvLEA/pGADT7 and pGBKT7, VvLEA/pGADT7 and pGBKT7, VvANR/pGBKT7 and pGADT7, pGADT7 and pGBKT7 were transformed into AH109 yeast strain, respectively. All the transformed yeast strains were grown on SD-4, both the C-VvLEA/pGADT7 and VvANR/pGBKT7 construct pair (Fig. 1A), and the VvLEA/pGADT7 and VvANR/pGBKT7 construct pair (Fig. 1B) had several positive clones, even when these clones were subcultured (Fig. 1C). The negative controls did not have any putative positive clones as expected (data not shown). For the CoIP of VvLEA and VvANR, C-VvLEA and VvANR in yeast respectively, anti-HA-tag antiserum (Bio-Med Biotechnology) was used to precipitate the proteins extracted from the transformed yeast, and detected VvANR/pGBKT7 with anti-MYC-tag antiserum (Bio-Med Biotechnology) (Fig. 1D, 1-2). The negative controls were the proteins extracted from the yeast co-transformed with VvLEA/pGADT7 and pGBKT7, C-VvLEA/pGADT7 and pGBKT7 for CoIP assays (Fig. 1D, 3-4), respectively. The CoIP showed that both VvLEA and C-VvLEA could interact with VvANR in yeast.

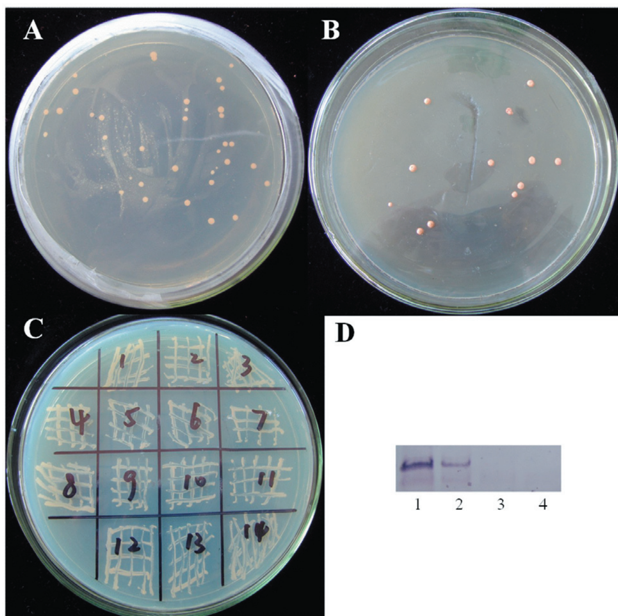

Fig. 1: Yeast two-hybrid (Y2H) and co-immunoprecipitation (CoIP) assays for the interaction of C-VvLEA and VvANR, VvLEA and VvANR with yeast total protein, respectively. **A**: Yeast co-transformed with C-VvLEA/pGADT7 and VvANR/pGBKT7 construct pair, grown on SD-4; **B**: Yeast co-transformed with VvLEA/pGADT7 and VvANR/pGBKT7 construct pair, grown on SD-4; **C**: The subculture of the positive clones in A and B, 1-7 for A, 7-14 for B; **D**: The CoIP of VvLEA and VvANR, C-VvLEA and VvANR in yeast, respectively. (1) for VvLEA/pGADT7 and VvANR/pGBKT7, (2) for C-VvLEA/pGADT7 and VvANR/pGBKT7, (3) for VvLEA/pGADT7 and pGBKT7, (4) for C-VvLEA/pGADT7 and pGBKT7.

The subcellular co-localization of VvANR and VvLEA: The co-localization of VvANR and VvLEA could be visualized in protoplasts of *A. thaliana*, which were transiently co-transfected with constructs for expression of VvANR-GFP and VvLEA-mCherry fusion proteins under control of CaMV 35S promoter. Localization of the red fluorescing VvLEA-mCherry fusion protein was detected under excitation with a 543 nm HeNe green laser (Fig. 2A), while VvANR-GFP was detected under excitation with a 488 nm argon-ion laser (Fig. 2B). When both the 543 nm HeNe green laser line and 488 nm argon-ion laser line were used to excite the fluorescence protein, the microscope captured the fluorescence from the co-transformed protoplasts. The co-localization of VvANR and VvLEA could be detected as yellow fluorescence (Fig. 2C). The *A. thaliana* protoplast co-transformed with VvANR-GFP and VvLEA-mCherry construct pair in bright field is shown in Fig. 2D. The result shows that VvANR can co-locate with VvLEA, properly in the cytoplasm, which is the essential condition for their interaction. It is reported that the GFP-vector does not ex-

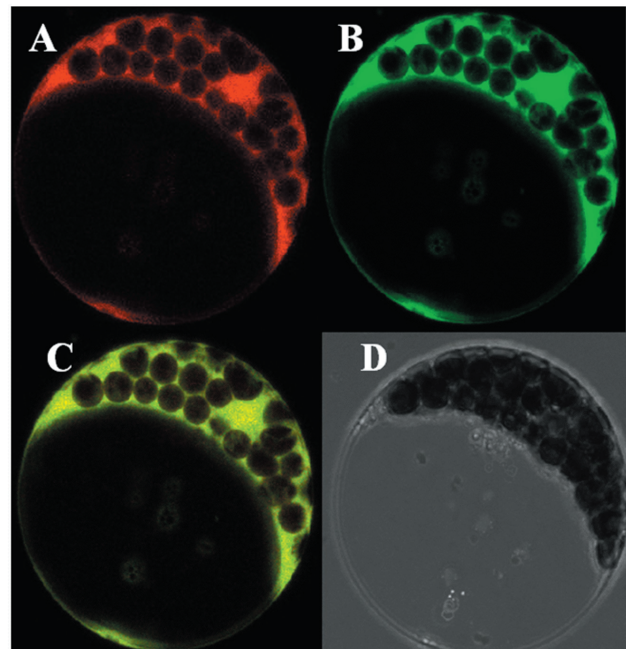

Fig. 2: The subcellular co-localization of VvANR and VvLEA in *A. thaliana* protoplast co-transformed with VvANR-GFP and VvLEA-mCherry construct pair. **A**: The localization of VvLEA-mCherry fusion protein; **B**: The localization of VvANR-GFP fusion protein; **C**: The co-localization of VvANR-GFP and VvLEA-mCherry fusion proteins; **D**: The co-transformed *A. thaliana* protoplast in bright field.

press GFP without adding coding sequence to the 5' end of the ORF of GFP (Zhao *et al.* 2011a), and we also did not detect any fluorescence of GFP in the control cells.

VvLEA interacted with VvANR tested by LCI: To further confirm the interaction between VvLEA and VvANR, LCI was used to verify it *in vivo*. This was achieved by *Agrobacterium*-mediated transient expression in *N. benthamiana*. We co-infiltrated VvLEA-CLuc and VvANR-NLuc, VvLEA-NLuc and VvANR-CLuc into a leaf of a *N. benthamiana* plant respectively (Fig. 3, I and II), and VvLEA-CLuc and NLuc, CLuc and VvANR-NLuc, CLuc and NLuc were added as negative controls into the same leaf (Fig. 3, III, IV and V). Both areas I and II showed Luc activity, while the negative controls could not reconstitute Luc enzyme to catalyze the luciferin reaction as expected, which further confirmed the interaction between VvANR and VvLEA *in vivo*.

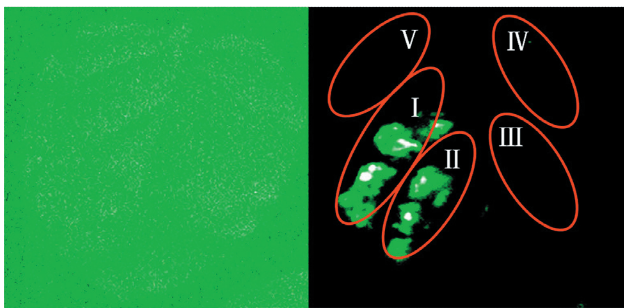

Fig. 3: VvLEA interaction with VvANR tested by firefly luciferase complementation imaging (LCI) system. The left panel shows the bright field image of the infiltrated tobacco leaf. The right panel shows the image that a CCD picture of imaging apparatus captured. I, II, III, IV and V were co-infiltrated with VvLEA-CLuc and VvANR-NLuc, VvLEA-NLuc and VvANR-CLuc, VvLEA-CLuc and NLuc, CLuc and VvANR-NLuc, CLuc and NLuc, respectively.

The relative expression of *VvANR* and *VvLEA* in 'Cabernet Sauvignon' berries under cold stress: The relative expressions of *VvANR* and *VvLEA* were examined in 'Cabernet Sauvignon during berry development with cold treatment. The two-fold change cutoff was used to define the differential expressions between the cold treated group and the corresponding control group (Fig. 4). The transcriptional activation of *VvANR* and *VvLEA* was time-course dependent.

For 'Cabernet Sauvignon' berries four weeks AFB, the expression of *VvANR* was just inhibited by cold treatment for 4 h, it was then up-regulated during the cold treatment. It reached a maximum at 20 h followed by a decrease by 28 h. *VvLEA* was induced at the transcriptional level by cold treatment, which reached up to three-fold of the corresponding control after 28 h of cold treatment. As the cold treatment time increased, the relative expression of *VvANR* was up-regulated in the eight-week AFB samples compared with the four-week AFB ones. The relative expression of *VvLEA* was also enhanced in the eight-week AFB samples, and showed a similar expression-response trend to those in the four-week AFB ones. In the 12-week AFB berries, *VvANR* was slightly induced by cold treatment for 4 h and 12 h, followed by a significant reduction. In contrast, cold stress promoted *VvLEA* expression, especially when the the treatment lasted more than 12 h.

About a two-fold up-regulation was observed for the relative expression of *VvANR* in four-week AFB berries after 20 h of cold treatment, in eight-week AFB berries after 28 h of cold treatment, and for the relative expression of *VvLEA* in four-week AFB berries after 28 h of cold treatment and in 12-week AFB berries after 20 and 28 h of cold treatment**.** The study shows that cold stress can inhibit the relative expression of *VvANR* in young berries after a short treatment period and in older berries after a longer treatment period. The up-regulation of the relative expression

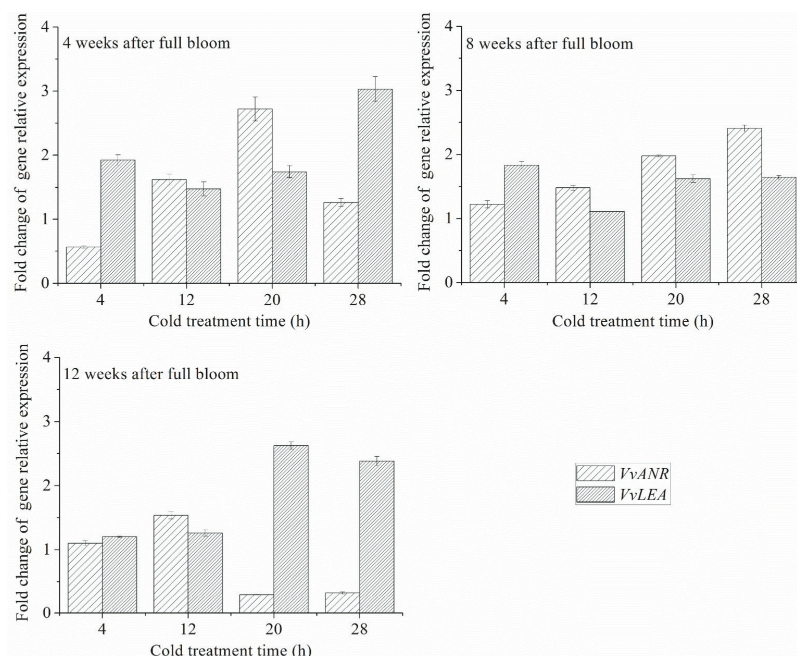

Fig. 4: The fold change of relative expression of *VvANR* and *VvLEA* in 'Cabernet Sauvignon' during grape development with cold treatment.

of *VvLEA* by cold treatment was in agreement with previous studies (Sutton *et al.* 1992). It has also been reported that LEA can protect the enzyme activity under cold stress (Liu *et al.* 2010, Zhao *et al.* 2011b). Here, we informed that VvLEA could protect VvANR activity under cold stress.

VvLEA: putative regulator and potential multi-enzyme complex member of PA biosynthesis: According to the previous studies, VvLEA we discovered in this study belongs to the LEA_2 group. LEA_2 was first found in cotton, which is an untypical LEA protein, it is hydrophobic but has no sequence homology with other LEAs (Galau *et al*. 1993). The LEA_2 found in *A. thaliana* contained one α-helix and seven β-strands, and could be induced by drought, cold, salt and light, but not by hormones like salicylic acid, jasmonic acid or ethylene. This indicates that LEA_2 is not a part of signal transduction pathways in plant-defense responses (Singh *et al*. 2005). LEA_2 is a group of cold-stress responsive genes, which can also stabilize cellular structures including proteins and membranes under dehydration, and can ameliorate the detrimental effects of ionic (KCl) or freezing stress, but not NaCl or osmotic stress in yeast cells (Cai *et al.* 1995, Campbell and Close 1997, Zhang *et al.* 2000, Soulages *et al.* 2003). It has been suggested that *VvMYB5b*, *VvMYBPA1* and *VvMYBPA2* can regulate *VvANR* at the transcriptional level (Bogs *et al*. 2007, Deluc *et al*. 2008, Terrier *et al*. 2009). However, the biological and non-biological factors having influence on these regulations from *VvMYB5b*, *VvMYBPA1* and *VvMYBPA2* remain unclear.

It has been reported that proteins might work in the form of multi-enzyme complex in the flavonoid pathway, especially for these having a direct interaction (Winkel-Shirley 1999, Jørgensen *et al.* 2005). Since ANR is an important enzyme in PA biosynthesis and LEA plays an important role in the stress response in plants, the interaction between VvLEA and VvANR, and the profiles of the relative expression of *VvANR* and *VvLEA* in developing grape berries under cold stress, may help us to infer the protection of VvANR from VvLEA under adverse conditions, as well as the protein-protein regulation and the formation of multi-enzyme complex in secondary metabolism in plants. To throw new light on these questions, our future research will focus on the mechanisms of protection and regulation of VvANR by VvLEA, study if and how VvLEA affects the enzyme activity of VvANR under stress conditions both *in vitro* and *in vivo*.

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References

Azuma, A.; Yakushiji, H.; Koshita, Y.; Kobayashi, S.; 2012: Flavonoid biosynthesis-related genes in grape skin are differentially regulated by temperature and light conditions. Planta **236**, 1067-1080.

Bais, H. P.; Vepachedu, R.; Gilroy, S.; Callaway, R. M.; Vivanco, J. M.; 2003: Allelopathy and exotic plant invasion: from molecules and genes to species interactions. Sci. Signal. **301**, 1377.

Bogs, J.; Downey, M. O.; Harvey, J. S.; Ashton, A. R.; Tanner, G. J.; Robinson, S. P.; 2005: Proanthocyanidin synthesis and expression of genes encoding leucoanthocyanidin reductase and anthocyanidin reductase in developing grape berries and grapevine leaves. Plant Physiol. **139**, 652-663.

Bogs, J.; Jaffe, F. W.; Takos, A. M.; Walker, A. R.; Robinson, S. P.; 2007: The grapevine transcription factor VvMYBPA1 regulates proanthocyanidin synthesis during fruit development. Plant Physiol. **143**, 1347-1361.

Boulton, R. B.; Singleton, V. L.; Bisson, L. F.; Kunkee, R. E.; 1995: Selection of state of ripeness for harvest and harvesting. In: R. B. Boulton (Ed.): Principles and practices of winemaking, 52-60. Chapman & Hall, International Thomson Publishing, New York, USA.

Cai, Q.; Moore, G. A.; Guy, C. L.; 1995: An unusual Group 2 LEA gene family in citrus responsive to low temperature. Plant Mol. Biol. **29**, 11-23.

Campbell, S. A.; Close, T. J.; 1997: Dehydrins: genes, proteins, and associations with phenotypic traits. New Phytol. **137**, 61-74.

Chen, H.; Zou, Y.; Shang, Y.; Lin, H.; Wang, Y.; Cai, R.; Tang, X.; Zhou, J. M.; 2008: Firefly luciferase complementation imaging assay for protein-protein interactions in plants. Plant Physiol. **146**, 368-376.

Chira, K.; Schmauch, G.; Saucier, C.; Fabre, S.; Teissedre, P. L.; 2009: Grape variety effect on proanthocyanidin composition and sensory perception of skin and seed tannin extracts from Bordeaux wine grapes (Cabernet Sauvignon and Merlot) for two consecutive vintages (2006 and 2007). J. Agric. Food Chem. **57**, 545-553.

Czemmel, S.; Heppel, S. C.; Bogs, J.; 2012: R2R3 MYB transcription factors: key regulators of the flavonoid biosynthetic pathway in grapevine. Protoplasma **249**, 109-118.

Czemmel, S.; Stracke, R.; Weisshaar, B.; Cordon, N.; Harris, N. N.; Walker, A. R.; Robinson, S. P.; Bogs, J.; 2009: The grapevine R2R3-MYB transcription factor VvMYBF1 regulates flavonol synthesis in developing Grape Berries. Plant Physiol. **151**, 1513-1530.

Deluc, L.; Barrieu, F.; Marchive, C.; Lauvergeat, V.; Decendit, A.; Richard, T.; Carde, J. P.; Mérillon, J. M.; Hamdi, S.; 2006: Characterization of a grapevine R2R3-MYB transcription factor that regulates the phenylpropanoid pathway. Plant Physiol. **140**, 499-511.

Deluc, L.; Bogs, J.; Walker, A. R.; Ferrier, T.; Decendit, A.; Merillon, J. M.; Robinson, S. P.; Barrieu, F.; 2008: The transcription factor VvMYB5b contributes to the regulation of anthocyanin and proanthocyanidin biosynthesis in developing grape berries. Plant Physiol. **147**, 2041-2053.

Dure III, L.; Greenway, S. C.; Galau, G. A.; 1981: Developmental biochemistry of cottonseed embryogenesis and germination: changing messenger ribonucleic acid populations as shown by *in vitro* and *in vivo* protein synthesis. Biochemistry **20**, 4162-4168.

Galau, G. A.; Hughes, D. W.; Dure, L.; 1986: Abscisic acid induction of cloned cotton late embryogenesis-abundant (Lea) mRNAs. Plant Mol. Biol. **7**, 155-170.

Galau, G. A.; Wang, H.; Hughes, D. W.; 1993: Cotton Lea5 and Lea14 encode atypical late embryogenesis-abundant proteins. Plant Physiol. **101**, 695.

Gris, E. F.; Mattivi, F.; Ferreira, E. A.; Vrhovsek, U.; Pedrosa, R. C.; Bordignon-Luiz, M. T.; 2011: Proanthocyanidin profile and antioxidant capacity of Brazilian *Vitis vinifera* red wines. Food Chem. **126**, 213-220.

He, F.; Fang, X. X.; Hu, M.; Pan, Q. H.; Shi, Y.; Duan, C. Q.; 2009: Preparation and biological application of antibodies against leucoanthocyanidin reductase and anthocyanidin reductase from grape berry. Vitis **48**, 69-75.

Hundertmark, M.; Hincha, D. K.; 2008: LEA (late embryogenesis abundant) proteins and their encoding genes in *Arabidopsis thaliana*. BMC Genomics **9**, 118.

Jørgensen, K.; Rasmussen, A. V.; Morant, M.; Nielsen, A. H.; Bjarnholt, N.; Zagrobelny, M.; Bak, S.; Møller, B. L.; 2005: Metabolon formation and metabolic channeling in the biosynthesis of plant natural products. Curr. Opin. Plant Biol. **8**, 280-291.

Jordão, A. M.; Gonçalves, F. J.; Correia, A. C.; Cantão, J.; Rivero-Pérez, M. D.; SanJosé, M. L. G.; 2010: Proanthocyanidin content, antioxidant capacity and scavenger activity of Portuguese sparkling wines (Bairrada Appellation of Origin). J. Sci. Food Agric. **90**, 2144-2152.

Jordão, A. M.; Simões, S.; Correia, A. C.; Gonçalves, F. J.; 2012: Antioxidant activity evolution during Portuguese red wine vinification and their relation with the proanthocyanidin and anthocyanin composition. J. Food Process. Preserv. **36**, 298-309.

Kennedy, J. A.; 2008: Grape and wine phenolics: Observations and recent findings. Cien. Inv. Agr. **35**, 107-120.

Koes, R.; Verweij, W.; Quattrocchio, F.; 2005: Flavonoids: A colorful model for the regulation and evolution of biochemical pathways. Trends Plant Sci. **10**, 236-242.

Liu, Y.; Zheng, Y.; Zhang, Y.; Wang, W.; Li, R.; 2010: Soybean PM2 protein (LEA3) confers the tolerance of *Escherichia coli* and stabilization of enzyme activity under diverse stresses. Curr. Microbiol. **60**, 373-378.

Marles, M.; Ray, H.; Gruber, M. Y.; 2003: New perspectives on proanthocyanidin biochemistry and molecular regulation. Phytochemistry **64**, 367-383.

Pan, Q. H.; Li, M. J.; Peng, C. C.; Zhang, N.; Zou, X.; Zou, K. Q.; Wang, X. L.; Yu, X. C.; Wang, X. F.; Zhang, D. P.; 2005: Abscisic acid activates acid invertases in developing grape berry. Physiol. Plant. **125**, 157-170.

Park, J. A.; Cho, S. K.; Kim, J. E.; Chung, H. S.; Hong, J. P.; Hwang, B.; Hong, C. B.; Kim, W. T.; 2003: Isolation of cDNAs differentially expressed in response to drought stress and characterization of the *Ca-LEAL1* gene encoding a new family of atypical LEA-like protein homologue in hot pepper (*Capsicum annuum* L. cv. Pukang). Plant Sci. **165**, 471-481.

Punta, M.; Coggill, P. C.; Eberhardt, R. Y.; Mistry, J.; Tate, J.; Boursnell, C.; Pang, N.; Forslund, K.; Ceric, G.; Clements, J.; Heger, A.; Holm, L.; Sonnhammer, E. L. L.; Eddy, S. R.; Bateman, A.; Finn, R. D.; 2012: The Pfam protein families database. Nucl. Acids Res. **40**, D290-D301.

Rao, L. J. M.; Yada, H.; Ono, H.; Ohnishi-Kameyama, M.; Yoshida, M.; 2004: Occurrence of antioxidant and radical scavenging proanthocyanidins from the Indian minor spice nagkesar (*Mammea longifolia* planch and triana syn). Bioorg. Med. Chem. **12**, 31-36.

Sano, T.; Oda, E.; Yamashita, T.; Naemura, A.; Ijiri, Y.; Yamakoshi, J.; Yamamoto, J.; 2005: Anti-thrombotic effect of proanthocyanidin, a purified ingredient of grape seed. Thrombosis Res. **115**, 115-121.

Shang, Y.; Yan, L.; Liu, Z. Q.; Cao, Z.; Mei, C.; Xin, Q.; Wu, F. Q.; Wang, X. F.; Du, S. Y.; Jiang, T.; Zhang, X. F.; Zhao, R.; Sun, H. L.; Liu, R.; Yu, Y. T.; Zhang, D. P.; 2010: The Mg-chelatase H subunit of Arabidopsis antagonizes a group of WRKY transcription repressors to relieve ABA-responsive genes of inhibition. Plant Cell **22**, 1909-1935.

Singh, S.; Cornilescu, C. C.; Tyler, R. C.; Cornilescu, G.; Tonelli, M.; Lee, M. S.; Markley, J. L.; 2005: Solution structure of a late embryogenesis abundant protein (LEA14) from *Arabidopsis thaliana*, a cellular stress-related protein. Protein Sci. **14**, 2601-2609.

Soulages, J. L.; Kim, K.; Arrese, E. L.; Walters, C.; Cushman, J. C.; 2003: Conformation of a group 2 late embryogenesis abundant protein from soybean. Evidence of poly (L-proline)-type II structure. Plant Physiol. **131**, 963-975.

Sutton, F.; Ding, X.; Kenefick, D. G.; 1992: Group 3 LEA gene *HVA1* regulation by cold acclimation and deacclimation in two barley cultivars with varying freeze resistance. Plant Physiol. **99**, 338-340.

Terrier, N.; Torregrosa, L.; Ageorges, A.; Vialet, S.; Verries, C.; Cheynier, V.; Romieu, C.; 2009: Ectopic expression of VvMybPA2 promotes proanthocyanidin biosynthesis in grapevine and suggests additional targets in the pathway. Plant Physiol. **149**, 1028-1041.

Winkel-Shirley, B.; 1999: Evidence for enzyme complexes in the phenylpropanoid and flavonoid pathways. Physiol. Plant. **107**, 142-149.

Zegzouti, H.; Jones, B.; Marty, C.; Lelievre, J. M.; Latché, A.; Pech, J. C.; Bouzayen, M.; 1997: ER5, a tomato cDNA encoding an ethylene-responsive LEA-like protein: characterization and expression in response to drought, ABA and wounding. Plant Mol. Biol. **35**, 847-854.

Zhang, L.; Ohta, A.; Takagi, M.; Imai, R.; 2000: Expression of plant group 2 and group 3 lea genes in *Saccharomyces cerevisiae* revealed functional divergence among LEA proteins. J. Biochem. **127**, 611-616.

Zhao, M. M.; Yang, B.; Wang, J. S.; Liu, Y.; Yu, L. M.; Jiang, Y. M.; 2007: Immunomodulatory and anticancer activities of flavonoids extracted from litchi (*Litchi chinensis* Sonn.) pericarp. Int. Immunopharmacol. **7**, 162-166.

Zhao, R.; Sun, H. L.; Mei, C.; Wang, X. J.; Yan, L.; Liu, R.; Zhang, X. F.; Wang, X. F.; Zhang, D. P.; 2011a: The Arabidopsis Ca^{2+}-dependent protein kinase CPK12 negatively regulates abscisic acid signaling in seed germination and post-germination growth. New Phytol. **192**, 61-73.

Zhao, X.; Zhan, L. P.; Zou, X. Z.; 2011b: Improvement of cold tolerance of the half-high bush Northland blueberry by transformation with the LEA gene from *Tamarix androssowii*. Plant Growth Regul. **63**, 13-22.

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