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Short communication

cDNA Clone, fusion expression and purification of the novel gene related to ascorbate peroxidase from Chinese wild *Vitis pseudoreticulata* in *E. coli*

Ling Lin†, Xiping Wang† & Yuejin Wang*
*Key Laboratory of Northwest Horticulture Plant Germplasm and Genetic Improvement of Ministry of Agriculture of People's Republic of China, College of Horticulture, Northwest A&F University, Yangling, Shaanxi, 712100, The People's Republic of China; *Author for correspondence (Phone: +86-29-87082803; E-mail: wangyj@public.xa.sn.cn; wangyuejin@263.net.)*

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Key words: cDNA clone, Chinese wild *Vitis* species, fusion protein expression, mRNA differential display, polyclonal antibodies

Abstract

Powdery mildew, caused by *Uncinula necator* Burr, is one of the most seriously damaging diseases of grapevine all over the world. To gain the novel gene and investigate the resistance mechanism in Chinese Wild *Vitis pseudoreticulata* clone Baihe-35-1, mRNA differential display was employed to study the differential expression of the resistant gene to the disease of it when inoculated by *Uncinula necator* under natural field conditions, 5′ RACE and 3′ RACE have been used to clone the whole cDNA sequences of VpAPX, the novel gene related to *Ascorbate Peroxidase* which involved in resistant to the disease, is composed of specific sequence 1077 bp and has an open reading frame of 750 bp coding for 250 amino acid residues with a molecular weight of 27.566 kDa. The VpAPX gene was obtained by polymerase chain reaction (PCR) with the special primers synthesized according to the sequences of cDNA, and further cloned it into the pGEM-T easy vector. The cloned VpAPX gene was cut out again with two restriction enzymes and was inserted into the prokaryotic expression vector pGEX-4T-1, then transferred into *E. coli* BL21. As result, GST-VpAPX fusion protein was successfully expressed by induction of IPTG and purified by GST affinity resin. After injecting rabbit, the polyclonal antibodies were produced. Western blot analyses showed that the antibody reacted specifically to GST-VpAPX fusion protein and the titer for this antibody is 10^5. This research made the foundation to transform the VpAPX gene into grape plants for follow research in processing.

Abbreviations: APX – ascorbase peroxidase; DDRT-PCR – differential display reverse transcript polymerase chain reaction; GST – glutathions-S-transferase; IPTG – isopropyl β-D-*thiogalactoside;* 5′ RACE – rapid amplification of 5′ cDNA end; 3′ RACE – rapid amplification of 3′ cDNA end

Introduction

Powdery mildew of grapevine, caused by *Uncinula necator* Burr, is the most economically important fungal disease of grapes (*Vitis vinifera* L.) worldwide, causing reduced yield and loss of wine quality. Control of powdery mildew on grapevine is currently achieved by the widespread application of fungicides such as sulphur and, more recently, systemic de-methylation inhibitor. While these

†These two authors contributed equally to this work.

chemicals are relatively effective, however the cost to the grower and the environmental impact of the residues remain undesirable. Like many other crops, utilization of host plant resistance through breeding would be an effective and economical strategy to control grape powdery mildew. Sources of resistant grape germplasm are needed as the foundation for such a breeding program [1]. Most of European grape cultivars were susceptible to powdery mildew but *V. labrusca* and Chinese *Vitis* grape species were resistant to the disease [2, 3]. China is one of the centers of origin of *Vitis* species [4]. In addition to their potential as sources of disease resistance, Chinese wild *Vitis* species do not have the foxy flavor that limits the use of some American native grapes in breeding programs [5]. Incorporating disease resistance genes from Chinese wild *Vitis* species into *V. vinifera* has been proven successful. However, utilization of the identical germplasm for cross breeding may take a significantly long time because of possible association of the resistant trait with undesired traits. Therefore, understanding the mechanisms of disease resistance and identifying key genes in the resistant germplasm could provide valuable information and resources for timely and efficient molecular breeding of new variety link resistant disease to excellent quality.

In the present study, mRNA differential display (DDRT-PCR) was employed to gain the gene of resistance to the disease in Chinese wild *V. pseudoreticulata* and we identified VpAPX, the ascorbate peroxidase related gene that are specifically induced by the fungus infection. 5′ RACE and 3′ RACE have been used to clone the whole cDNA sequences of VpAPX (GenBank Accession No. DQ150258).

According to the sequences of the cDNA to design and synthesize special primers, the VpAPX gene was obtained by polymerase chain reaction (PCR), and then inserted it into prokaryotic expression vector. The GST-VpAPX fusion protein was successfully expressed after the recombinant vector being induced with isopropyl β-D-thiogalactoside (IPTG). Selected the optimal expression condition and analyzed the solubility of the fusion protein in the research, the purity fusion protein was obtained after using GST affinity resin purified the soluble protein. After injecting adult rabbit with the fusion protein, polyclonal antibodies were produced.

Materials and methods

Plant material and treatments

The grape material of Chinese wild *V. pseudoreticulata* clone Baihe-35-1 highly resistant to powdery mildew and *V. adstricta* Hance clone Taishan-2 highly susceptible to powdery mildew maintained in the grape germplasm resources orchard [2], Northwest A&F University, Yangling Shaanxi, 712100, People's Republic of China, was used for the present study. The inoculation was carried out under natural field conditions by pressing infected leaves with *Uncinula necator* of Taishan-2 against the uninfected leaves of Baihe-35-1 pre-sprayed with sterile water from 8:00 am to 10:00 am on August 12, 2002. The control is only sprayed with sterile water on the young leaves of Baihe-35-1, the inoculated leaves were immediately covered with paper bags.

Bacterial strains and plasmids

Escherichia coli strains DH5α and BL21 were used as the hosts for subcloning and expression, respectively, purchased from Amersham Biosciences, USA. The pGEM-T easy vector (Promega, USA) was used as the vector for subcloning of PCR product of VpAPX gene and pGEX-4T-1 (Amersham Biosciences, USA) was used for expression.

Enzyme and reagents

DNA polymerase and T_4 DNA ligase were purchased from Shanghai Sangon Co (Shanghai, China). The restriction enzymes were purchased from Takara Biotechnology Co (Japan). The plasmid extraction kit and DNA agarose gel cleanup kit were purchased from Tian Wei Shi Dai Biotechnology Co (Beijing, China).

Total RNA isolation and mRNA differential display reverse transcript-PCR (DDRT-PCR)

Total RNA was isolated from the grape leave samples after 0, 1, 3, 5, 7 and 9 d treatment by the method of SDS/phenol [6] with some modifications. mRNA differential display was performed as described by Liang and Pardee [7] and in reverse transcription reactions primed with the

oligonucleotide 5′-TTTTTTTTTTTGG-3′ (T_{11}GG). The reaction conditions for reverse transcription (20 μl) were: 1.0 μg total RNA, 6.0 mM anchor primer T_{11}AC, 2.5×10^{-4} mM dNTPs, 20 U RNasin, 5× transcription buffer 4.0 μl and 200 U M-MMLV transcriptase (Promega, USA). Reactions were incubated at 70 °C for 5 min, cooled in ice and 20 U RNasin, 200 U M-MMLV transcriptase added, then incubated for 60 min at 37 °C and 95 °C for 5 min. PCR reactions (25 μl) were performed with 1.0 μl RT reaction first strand, 2.5×10^{-3} mM anchor primer T_{11}AC, 2.5×10^{-3} mM arbitrary primer (S421∼S440, Sangon, China), 0.25 mM dNTPs, 1.0 U *Taq* DNA polymerase. PCR reaction conditions were: 94 °C for 1 min, 40 cycles of 94 °C for 30 s, 40 °C for 2 min, 72 °C for 30 s and a final extension step at 72 °C for 10 min. 4.5 μl product of DDRT-PCR was separated on 6% polyacrylamide sequencing gel for 3.5 h at 2000 v. To detect false-positive, both reverse transcription and PCR reactions were carried out in duplicate for each sample. The results of DDRT-PCR were analyzed by a silver-stain method [8] with the following modifications. The gel was fixed in 10% acetic acid for 30 min, rinsing with H_2O and incubated with solution A (1 g/l $AgNO_3$, 1.5 ml/l 37% formaldehyde) at 37 °C for 30 min, rinsed 2–3 times with H_2O, developed in solution B (30 g/l Na_2CO_3, 1.5 ml/l 37% formaldehyde, 2 mg/l $Na_2S_2O_3 \cdot 5H_2O$) and fixed in solution C (10% acetic acid). The gel was then washed twice with H_2O and air-dried at the room temperature. The cDNA bands of interest were excised from the dried gel with a scalpel. Gel slices were heated in 30 μl distilled water at boiling temperature for 10 min to elute cDNAs and used directly for PCR re-amplification at the annealing temperature 42 °C. The products of re-amplification were cloned into pGEM-T easy vector (Promega, USA), followed by sequencing.

5′ RACE and 3′ RACE end PCR amplification of the VpAPX cDNA from V. pseudoreticulata

In order to obtain the full-length sequence of the cDNA, a rapid amplification of 3′ cDNA end (3′ RACE) and 5′ end (5′ RACE) PCR amplification were carried out. The gene specific primer GSP1: 5′-GACAAGAGGACGGAAGGCTGGGTC-3′ for 5′RACE and GSP2: 5′-TGCCAGATGCCACCAAAGGATGC-3′ for 3′ RACE were designed based on the sequence of the differential cDNA fragment T_{11}GG/S438-499. The length between the two specific primers was 272 bp. Total RNA of Baihe-35-1 leaves inoculated with *Uncinula necator* after 7 d was isolated as above described and RACE was performed according to the manufacturer's instructions (BD SMART™ RACE cDNA Amplification Kit). The 5′ RACE and 3′ RACE products were separated by the 1.2% agarose gel electrophoresis and purified with the columns (Sangon, China). The 5′ RACE and 3′ RACE cDNA fragments were cloned into pGEM-T easy vector, and sequenced by Takara Biotechnology Co. Lt.

Sequence analysis

Nucleotide and amino acid sequences of VpAPX were compared with those in GenBank databases by using the BLAST analysis program. The alignment and phylogenetic reports were produced using DNAMAN.

Construction of VpAPX gene in the expression vector pGEX-4T-1

Using cDNA from RT-PCR as template, the VpAPX gene was amplified with primer 1 (5′-GCGGATCCATGGAAGAGCTATCCTAC-3′) and primer 2 (5′-CGCTCGAGTTAGGCAT CAGCAAATCC -3′). The sequences underlined are the recognition sites of the restriction enzymes *Bam*HI and *Xho*I, respectively. PCR reaction conditions were: 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 65 °C for 2 min, 72 °C for 30 s and a final extension step at 72 °C for 5 min. The amplified PCR products were ligated into the pGEM-T easy vector, resulting in pGEM-T/VpAPX. Positive clone was confirmed by restriction enzyme digestion. The VpAPX fragment was isolated from the pGEM-T easy vector by digestion with *Bam*HI and *Xho*I. After the VpAPX fragment being ligated into the pGEX-4T-1 vector that digested by the same enzymes to obtain an in-frame fusion gene. The recombinant vector pGEX-4T-1 was identified by restriction digestion and the VpAPX insert was verified by sequencing. The correct recombinant prokaryotic expression vector was named as pGEX-4T-1/VpAPX. *E.coli* DH5α was used for amplification of the recombinant plasmid pGEM-T/VpAPX and

E. coli BL21 was transformed for inducing expression of GST-VpAPX fusion protein.

Expression of the GST-VpAPX fusion protein

The transformat in culture medium containing 100 mg/l ampicillin was grown to $OD_{600} = 0.6 \sim 0.8$. Then, IPTG was added to the final concentration of 0.3 mM. After induction at 37 °C for 5 h in the conical flask, the bacteria were collected by centrifugation at 10,000 × *g* for 1 min and then were lysed by boiling in SDS-PAGE sample buffer for 3–5 min. The results were analyzed by 12% SDS-PAGE. After obtained the correct prokaryotic expression plasmid, following is selected the optimal expression condition of GST-VpAPX fusion protein. The prokaryotic expression plasmids were induced with IPTG of different concentration (0.1, 0.2, 0.3, 0.4, 0.5, 0.6 mM) at different temperature (20, 25, 30, 37 °C) for different time (1, 2, 3, 4, 5, 6, 7 h), respectively, the bacteria were harvested by centrifugation and to be lysed like before. The results were analyzed by 12% SDS-PAGE.

Purification of GST-VpAPX fusion protein

A single colony of *E. coli* BL21 cells transformed with the recombinant expression plasmid pGEX-4T-1/VpAPX were inoculated in flask containing 20 ml of LB medium with 100 mg/l ampicillin, and cultured it overnight at 37 °C in a shaking incubator. Transferred 1 ml of the culture to a 250-ml flask containing 100 ml of the same medium. The flask was shake at 37 °C until the OD_{600} of the culture was about 0.6∼0.8. Then, IPTG was added to the final concentration of 0.1 mM, after induction at 25 °C for 4 h, the cells were collected by centrifugation at 12,000 × *g*, at 4 °C for l0 min. The supernatant was discarded and the cell pellet was lysed in the SuperE Lysis Solution (Da Bodi Bio, Beijing) for 5–l0 min at room temperature. The lysed cells were centrifuged at 12,000 × *g*, at 4 °C for 10 min. Then, 5 ml of the 50% slurry of glutathione-Sepharose was added into the supernatant liquid. After incubation with gentle agitation at room temperature for 30 min, the suspension was centrifuged at 500 × *g*, at 4 °C for 5 min to sediment the matrix. The supernatant was then discarded. After washing the glutathione-Sepharose 4B pellet with 10 bed volumes of PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3), the suspension was centrifuged at 500 × *g*, at 4 °C for 5 min to sediment the matrix. The wash was repeated more than twice. After that, 5 ml elution buffer (10 mM reduced glutathione in 50 mM Tris–HCl, pH 8.0) was added into the sedimented matrix of glutathione-Sepharose 4B per milliliter. The matrix was then mixed gently and incubated at room temperature for 10 min to elute the bound matrix. The supernatant was then transferred into a fresh centrifuge tube. The elution was repeated and centrifuged for a total of three times and the eluates were mixed together. The results were analyzed by 12% SDS-PAGE.

Production of polyclonal antibody

The concentration of protein was analyzed by the way of Brodford, then the total bacterial protein was resolved on 12% SDS-PAGE, the GST-VpAPX fusion protein was cut, grined it into powder and lysed it in physiological salt solution. The solution mixed with Freund's complete adjuvant, and the GST-VpAPX fusion protein was about 1–2 mg/ml, the adult rabbit was subcutaneously injected with 0.5 ml each time a week for a total of four times, then the solution mixed with Freund's uncomplete adjuvant and injected to the rabbit. About 10 days later, killed the rabbit to take the total blood, the polyclonal antibody was obtained.

Western blotting to identify the specificity of polyclonal antibody

Using BSA as negative control, the purified protein and the total bacterial protein as positive control, the entire protein sample analyzed by 12% SDS-PAGE. After electrophoresis, the gel was immersed in the transfer buffer (0.24% Tris–HCl, 1.153% glycine, and 20% methanol, pH 8.8) for 30 min, and then the protein was transferred onto nitrocellulose membrane by electrophoresis at 100 v for 2 h. The membrane was incubated for 1 h in blocking buffer (15% skim milk, 100 mM Tris–HCl, pH 7.5, 0.9% NaCl, and 0.1% Tween-20). Then the membrane was incubated with the anti-GST-VpAPX polyclonal antibody for 1 h. After being washed three times (each time for 15 min) with TBS–Tween buffer (100 mM

Tris–HCl, pH 7.5, 0.9% NaCl, and 0.1% Tween-20), the membrane was incubated for 1 h with alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG. After being washed three times (each time for 15 min) with TBS-Tween buffer, the specific protein bands were visualized after staining by 5-bromo-4-chloro-3-indoxyl phosphate/nitroblue tetrazolium (BCIP/NBT), the reaction was stopped by washing the membrane in distilled water. Isoforms of peroxidase was extracted from Chinese wild *V. pseudoreticulata* Baihe-35-1 which was inoculated by *Uncinula necator*. The protein sample analyzed by 12% SDS-PAGE and Western blotting like before.

Results

mRNA Differential display

Total RNAs from leaves of *V. pseudoreticulata* clone Baihe-35-1 after 0, l, 3, 5, 7, 9 d treatment were extracted, reverse-transcribed with anchor primer $T_{11}GG$, followed by PCR with 20 combinations of anchor primer $T_{11}GG$ and 20 random primers. All the RT-PCR reactions were carried out and analyzed in duplicate in order to minimize spurious results. cDNA fragment $T_{11}GG$/S438-499, amplified with the anchor primer $T_{11}GG$ and the random primer S438 5′-GGTGAGGTCA-3′ were expressed more strongly in leaves after inoculated with *Uncinula necator* 1, 3, 5, 7 and 9 d than in control leaves of Baihe-35-1 from all primer combinations [9]. Sequence analysis revealed that this fragment actually was made up specific nucleotide sequence 449 bp in length and its nucleotide sequence shared highly identity with *Spinacia oleracea, Oryza sativa, Zea mays, Nicotiana tabacum, Zantedeschia aethiopica* APX released in GenBank databases, implying that it was probably a part of APX gene.

5′ RACE and 3′ RACE end PCR amplification of the VpAPX cDNA from V. pseudoreticulata

5′ RACE and 3′ RACE were employed to obtain the full-length cDNA sequence of VpAPX gene. A total of 6.0 μl aliquots of the amplification products were separated on 1.2% agarose gel after the RACE amplification. The 5′ RACE product showed about specific sequence 750 bp (Figure 1).

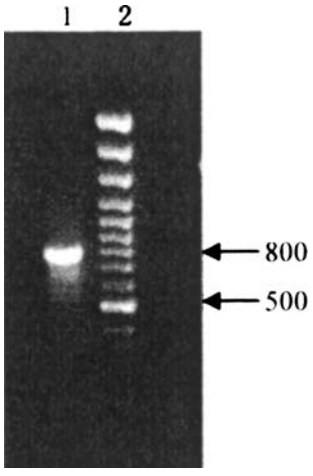

Figure 1. Schematic of the 1.2% agarose gel electrophoresis of 5′ RACE cDNA of *V. pseudoreticulata* clone Baihe-35-1 VpAPX. Lane 1, 5′ RACE cDNA; lane 2,100 bp DNA ladder.

Then it was cloned into pGEM-T easy vector, transformed into *E. coli* DH5α. The positive clone, identified by blue/white screening and *Eco*RI digest, was sequenced and it was actually composed of specific sequence 733 bp. The 3′ RACE products demonstrated about 650 bp (Figure 2), and it was actually 616 bp in length sequenced by Takara Biotechnology Co. Lt.

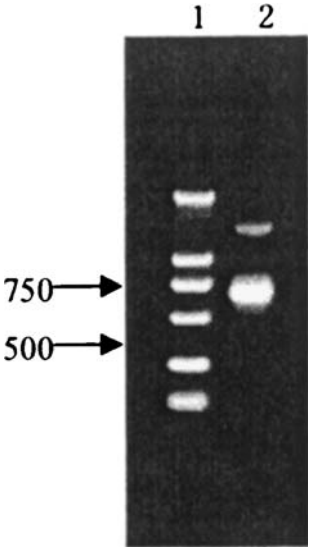

Figure 2. Schematic of the 1.2% agarose gel electrophoresis of 3′ RACE cDNA of *V. pseudoreticulata* clone Baihe-35-1 VpAPX. Lane 1, DNA marker DGL2000; lane 2, 3′ RACE cDNA.

Sequence analysis

One cDNA with full-length of 1077 bp was produced because of 272 bp overlapping sequences of 5′ and 3′ end fragment, which is designated as VpAPX. VpAPX contains an open reading frame and contains a 70 bp 5′ UTR (Untranslated region) and a 254 bp 3′ UTR that includes the putative polyadenylation signal AATAAA at position 1030 nt and a polyA tail [9]. The sequence is 250 residues long with a molecular weight of 27.566 kDa and was found to have conserved amino acids of APX, H42 (active site), H163 (binding site), D207 (active site), W179 (active site) [9]. These amino acids are known to make up part of the His-Asp-Trp catalytic triad, as determined from the three-dimensional structure [10].

To detect similarities and differences in individual amino acid sequence positions, we aligned the deduced amino acid sequence of VpAPX with the sequence of other plant APX in the database of NCBI Blast search [9]. The deduced amino acid sequence of VpAPX had an 79% identity with that of *Hevea brasiliensis* (GenBank Accession No. AF457210), an 82% identity with that of *Glycine max* (GenBank Accession No. AB082932), an 83% identity with that of *Arabidopsis thaliana* (GenBank Accession No. NM_100663), an 84% identity with that of *Spinacia oleracea* (GenBank Accession No. L20864), an 85% identity with that of *Oryza sativa* (GenBank Accession No. AB050724) and *Zea mays* (GenBank Accession No. Z34934), an 86% identity with that of *Nicotiana tabacum* (GenBank Accession No. U15933), and an 88% identity with that of *Zantedeschia aethiopica* (GenBank Accession No. AF053474) APX [9]. The fact that the cloned cDNA does not have transit peptide supports the idea that this enzyme exists in the cytosol [11].

Construction of pGEX-4T-l/VpAPX

The VpAPX gene was amplified by PCR with a pair of primers templated by cDNA from RT-PCR. As shown in Figure 3, the PCR product was about 780 bp, which was consistent with the theoretical length of VpAPX gene. The recombinant plasmid pGEM-T/VpAPX was digested by the restriction enzymes *Bam*HI and *Xho*I. Agarose gel electrophoresis of the digest, revealed a DNA band

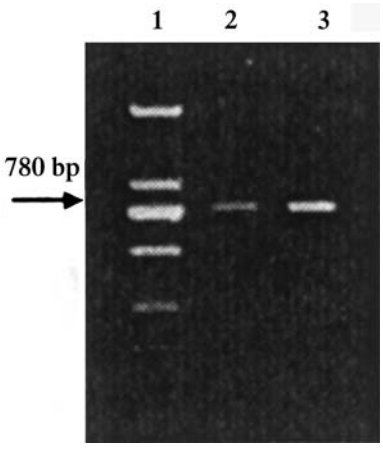

Figure 3. The results of agarose gel electrophoresis of PCR product. Lane 1, DNA marker; lanes 2 and 3, PCR products.

at about 780 bp (Figure 4). After inserted the VpAPX gene into expression vector pGEX-4T-1, the recombinant plasmid pGEX-4T-1/VpAPX was digested by the restriction enzymes *Bam*HI and *Xho*I. Agarose gel electrophoresis of the digest, a DNA band at about 780 bp was detected (Figure 5). The inserted fragment was verified by sequencing and the result was identical to the VpAPX gene cloned from the Chinese wild *Vitis pseudoreticulata* clone Baihe-35-1 and in the correct reading frame with the GST-tag in the vector.

Expression of the GST-VpAPX fusion protein

The GST-VpAPX fusion protein was successfully expressed by inducing the tac promoter with IPTG. Cells of *E. coli* BL21 transformed with

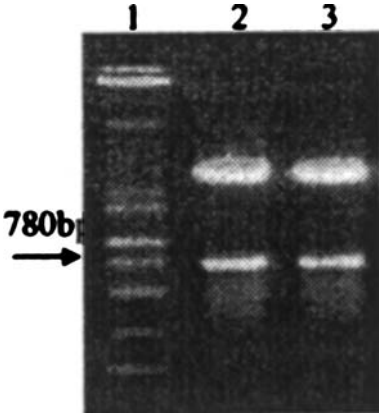

Figure 4. Identification of recombinant plasmid pGEM-T/VpAPX. Lane 1, DNA marker; lanes 2 and 3, pGEM-T/VpAPX candidates digested by *Bam*HI and *Xho*I.

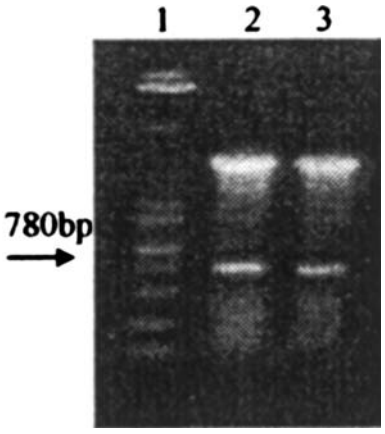

Figure 5. Detection of recombinant plasmid pGEX-4T-1/VpAPX. Lane 1, DNA marker; lanes 2 and 3, pGEX-4T-1/VpAPX candidates digested by *Bam*HI and *Xho*I.

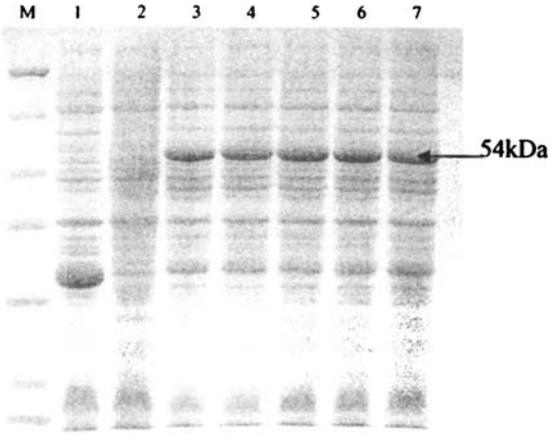

Figure 6. Identification of the GST-VpAPX fusion protein induced by different concentration of IPTG. M, protein marker; lane 1, pGEX-4T-1 induced by 0.3 mM IPTG for 7 h; lane 2, uninduced pGEX-4T-1/VpAPX; lanes 3–7, pGEX-4T-1/VpAPX induced by IPTG with serial concentration of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 mM for 7 h, respectively.

pGEX-4T-1 vector and induced with IPTG produced a protein of 26 kDa (GST protein). Similarly, cells transformed with pGEX-4T-1/VpAPX plasmid and induced with IPTG produced a protein of around 54 kDa (GST-VpAPX fusion protein) that was absent in non-induced cells. The expression level of GST-VpAPX fusion protein did not change with the increasing of concentration of IPTG (Figure 6), but it increased with increasing induction time. The fusion protein was best produced after IPTG induced for 4 h (Figure 7). The fusion protein accounts for 35% of the total bacterial protein by analyzed the bands with Band

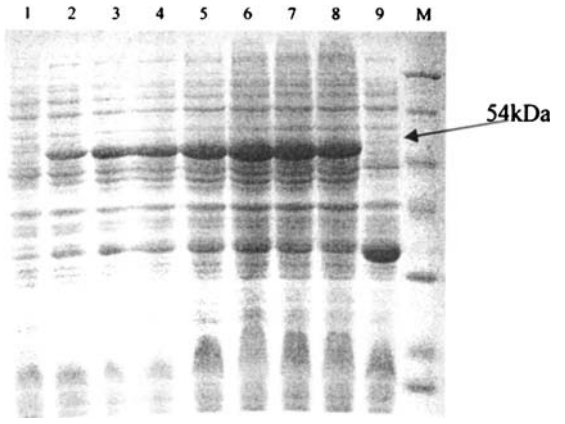

Figure 7. Expression of GST-VpAPX fusion protein for different time. M, protein marker; lane 1, uninduced pGEX-4T-1/VpAPX; lane 9, pGEX-4T-1 induced by 0.3 mM IPTG; lanes 2–8, pGEX-4T-1/VpAPX induced by 0.1 mM IPTG for l, 2, 3, 4, 5, 6, 7 h, respectively.

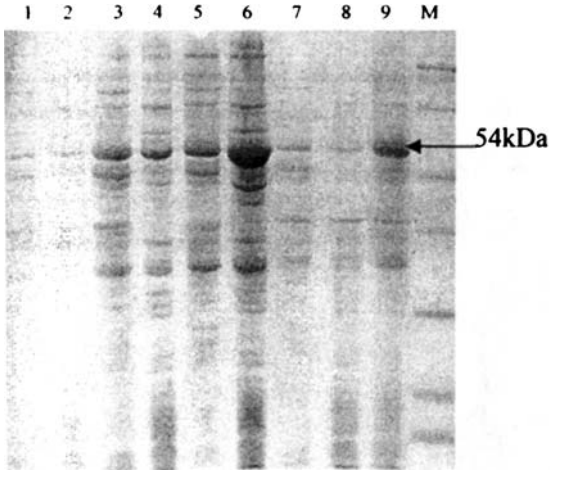

Figure 8. Expression of GST-VpAPX fusion protein at different temperature. M, protein marker; lane 1, sediment induced at 20 °C; lane 2, supernatant induced at 20 °C; lane 3, sediment induced at 37 °C; lane 4, sediment induced at 25 °C; lane 5, supernatant induced at 37 °C; lane 6, total protein induced at 37 °C; lane 7, sediment induced at 30 °C; lane 8, supernatant induced at 30 °C; lane 9, supernatant induced at 25 °C.

Scan program. As shown in Figure 8, at different temperature, all the fusion protein had two kinds of forms – soluble protein and inclusion body, but after induced at 25 °C, the soluble protein reached the highest level when compared with the rest.

Purification of GST-VpAPX fusion protein

The cells of 100 ml culture were collected by centrifugation. The obtained cell pellet was lysed

in 5 ml SuperE Lysis Solution for 5–10 min at room temperature. The bacterial lysate, after clarification by centrifugation, was incubated with GSH-Sepharose for 30 min. The resin was then washed with PBS buffer for three times. The fusion protein bound to the resin eluted with 10 mM GSH solution. As results showed in Figure 9, purity fusion protein was obtained.

Specificity of the polyclonal antibody

As shown in Figure 10, when the dilutions of polyclonal antibody were 10^1, 10^2, the bands of Western blotting were not clear. When increased the dilution to 10^3, 10^4, 10^5, the bands after dyeing became clearer. So the polyclonal antibody with a titer of 1:1000 was chosen for further analysis. Western blot (Figure 10) showed the GST-VpAPX could be recognized by the polyclonal antibody against the fusion protein. However, for the negative control BSA, no obvious band was detected. For the isoforms of peroxidase extracted from grape leaves, there was only one obvious band was detected (Figure 11). These results indicated that the polyclonal antibody had high specificity for GST-VpAPX fusion protein and APX from Chinese wild grape leaves.

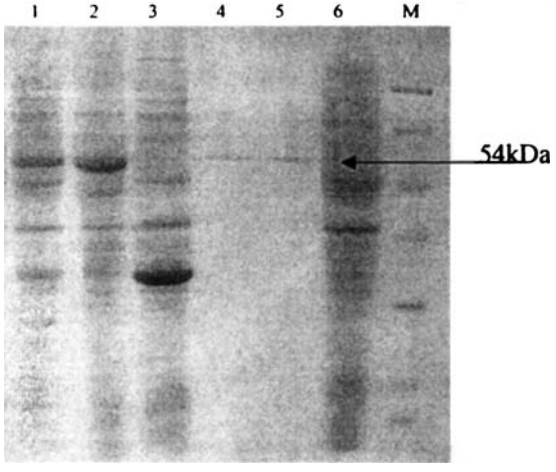

Figure 9. Purification of GST-VpAPX fusion protein by glutathione-Sepharose. M, protein marker; lane 1, sediment induced at 25 °C; lane 2, supernatant induced at 25 °C; lane 3, pGEX-4T-1 induced at 37 °C; lanes 4 and 5, two purified protein; lane 6, uninduced pGEX-4T-1/APX.

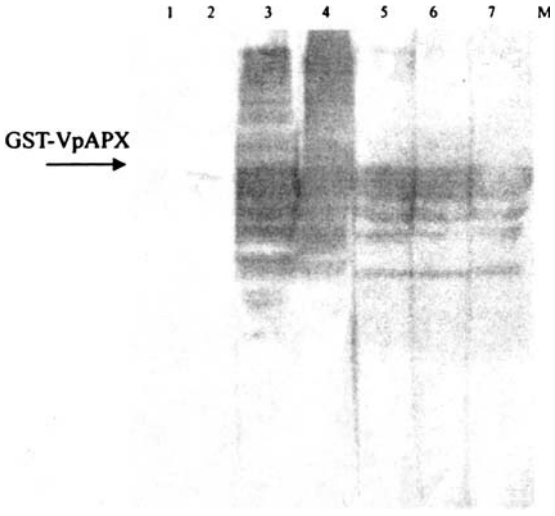

Figure 10. Detection of the specificity of polyclonal antibody to GST-VpAPX by Western blotting. M, protein marker; lane 1, negative control BSA; lane 2, purified protein reacted with polyclonal antibody; lanes 3–7, the total protein react to polyclonal antibody with serial dilution of 10^1, 10^2, 10^3, 10^4, 10^5.

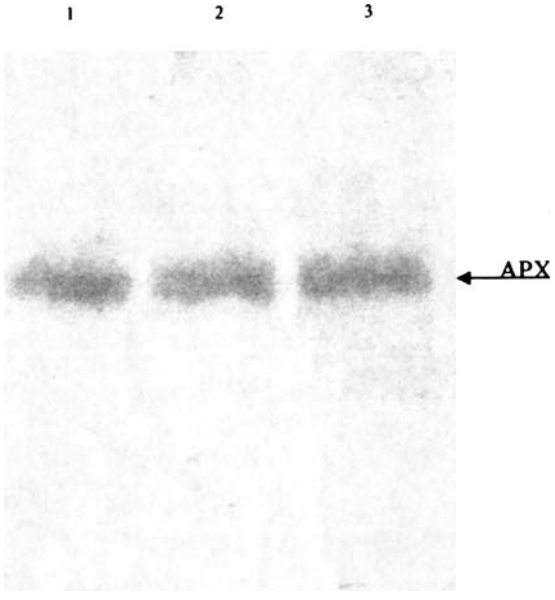

Figure 11. Detection of the specificity of polyclonal antibody to APX by Western blotting. Lane 1, APX from uninoculated grape leaves; lane 2, APX from grape leaves inoculated with *Uncinula necator* for 7 d; lane 3, APX from grape leaves inoculated with *Uncinula necator* for 12 d.

Discussion

At low temperature, drought, air pollution and pathogen invasion, the plant cells will produce a lot of reactive oxygen species (ROS) [12], such as

superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl (OH^-) and single oxygen ($^1O_2$). These oxygenic compounds are very active, they can destroy normal metabolism of cells by act on DNA, protein, lipids and sugars [13], thus cause oxidizing stress to the plants. There are two antioxidative systems in plant cells. One way needs catalysis of enzyme and the other way does not need [14]. The system of enzyme catalyzed includes *Superoxide dismutase* (SODs), *Ascorbate Peroxidase* (APXs), *Glutathion reductase* (GR) and Catalase (Cat). The other includes glutathions, flavonoids and ascorbic acid. APX is the key enzyme that scavenges H_2O_2 in plant cells, and has high affinity to H_2O_2 [15–18]. It anti-oxidize by resolve H_2O_2 into H_2O when the ascorbic acid existed. In higher plants, APX exists in at least three forms: cytosolic, stromal and thylakoid membrane-bound isoforms. At present there are many reports about cytosolic APX, like in *Spinach, Arabidopsis thaliana* [19], *soybean* [20], potato tubers [21], strawberry fruit [22] and *Pea* [11]. But there is no report on the grapevine, especially in Chinese wild grape related to APX gene. In our study, 5′ RACE and 3′ RACE have been used to clone the whole cDNA sequences of VpAPX from Chinese wild *Vitis pseudoreticulata*. So that we have obtained the novel gene related to APX, then made it expressed in *E. coli* and analyzed its peculiarity of expression and biological function.

In the present study, a cDNA fragment T_{11}GG/S438-499 of VpAPX gene were expressed more strongly in leaves after treatment 1, 3, 5, 7 and 9 d than in control leaves but the quantity of protein was not changed, this result was consistent with the result of Shen's study [18]. We cloned the cDNA of VpAPX from *V. pseudoreticulata*. The full-length sequence of VpAPX was composed of specific sequence 1077 bp and contained an ORF of 750 bp, which was large enough to encode APX. In deduced amino acid sequence, it shared nearly 80–90% similarity with other plant APX. In this research, the glutathions-S-transferase system was selected as the fusion expression vector, the GST fusion protein are widely used in protein production for pure immunogens, protein–protein, and DNA–protein interaction studies [23–26]. Every pGEX vector has an open reading frame coding GST, later is the mono-restriction enzyme sites, following is the stop codes corresponding to the open reading frame. The GST has lower immunity, but we can purify protein easily by special combination between GST and glutathione-Sephrose. In the study, we have successfully produced the GST-VpAPX fusion protein in *E. coli* BL21 with high yield. Furthermore, we explored the influence that different temperature brought about to the solution of fusion protein. Although at the normal temperature the bacteria grow (at 37 °C), the fusion protein forms soluble protein partly, with the change of temperature, the soluble protein was increased at 25 °C. Probably, the bacteria have slow metabolism at lower temperature, so the protein expressed could not form inclusion body so easily [25]. Purity protein was obtained after the soluble protein purified by glutathione-Sephrose.

After injecting adult rabbit with GST-VpAPX fusion protein, polyclonal antibody with high titer and reacted with antigen peculiarly was produced. Therefore, we can use Western blot with this antibody to detect the expression level of VpAPX gene in grape plants and analyze its expression in transgenic plants. Furthermore, we can purify this antibody by the way of ammonium sulfate precipitation and ion exchange chromatography (IEC). The purified antibody made it is possible to locate the VpAPX gene in plant cells with Electromicroscopy immunogold labeling and to analyze the interaction between APX and other protein with immunoprecipitation or other immunological methods, also can elucidate the biological function of VpAPX gene and molecular mechanism of its resistance to the disease. The novel gene of Chinese wild grape obtained in this research is being transformed into European grape cultivars as the gene of interest resistant to the disease for next study.

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