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Full Length Research Paper

Expression analysis of a pathogen-responsive PR-1 gene from Chinese wild

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In response to pathogen attacks, plant produces a wide range of pathogenesis-related (PR) proteins. PR-1 genes represent the first identified PR gene family. Most members of PR-1 gene family are not inducible by pathogen attacks. In this study, we identified a pathogen-responsive PR-1 gene designated as *VqPR-1* (GenBank accession no. JN256202), in a subtractive suppression hybridization (SSH) cDNA-library from *Elsinoe ampelina*-inoculated young leaves of Chinese wild *Vitis quinquangularis* clone 'shang-24'. *VqPR-1* protein contained the requisite signal sequence at the N-terminus, a conserved three-dimensional structure called 'PR-1 fold' and a highly conserved six-cysteine motif. Expression level of *VqPR-1* rose rapidly in response to *E. ampelina* infection. The three tested plant defence signaling molecules, salicylic acid (SA), ethephon (Eth) and methyl jasmonate (MeJA) all triggered an induction of *VqPR-1*. However, the induction by addition of MeJA was weaker than that induced by SA and Eth. In addition, the response to inoculation with *E. ampelina* or treatment with signaling molecules, was sometimes a suppression of *VqPR-1* gene expression. The highest expression of *VqPR-1* was observed in flowers, stems and leaves, while low-level or no obvious transcripts were detected in pericarps and tendrils, respectively.

Key words: *Vitis quinquangularis*, PR-1 gene, *Elsinoe ampelina*, expression analysis.

INTRODUCTION

Plants and pathogens have continuously confronted each other during evolution in a battle for growth and survival (Lopez et al., 2008). Plants have evolved numerous sophisticated mechanisms to protect themselves against pathogens. Some of these defense mechanisms are constitutive, while others are induced upon pathogen attack. Systemic acquired resistance (SAR) is an inducible defense response that can be invoked by a specific pathogen, and leads to resistance against subsequent challenges by a wide range of pathogens (Delaney et al., 1994). SAR is associated with the activation of a large number of genes encoding various types of stress proteins, including pathogenesis-related (PR) proteins (Enkerli et al., 1993; Ward et al., 1991). PR proteins have been defined as proteins encoded by the host plant but induced only in pathological or related situations (Antoniw

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Abbreviations: SSH, Subtractive suppression hybridization; **qRT-PCR,** quantitative reverse transcription-polymerase chain reaction; **sqRT-PCR,** semi-quantitative reverse transcription-polymerase chain reaction; **SAR,** systemic acquired resistance; **PR,** pathogenesis-related; **SA,** salicylic acid; **MeJA,** methyl jasmonate; **Eth,** ethephon; **Hpi,** hours post inoculation; **Hpt,** hours post treatment.

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and White, 1980). Induction of PR genes has been found in many plant species from both dicot and monocot species belonging to various families (Sels et al., 2008; Van Loon, 1999; Van Loon et al., 2006), suggestive of a general role for PR proteins in plants' adaptation to biotic stress conditions. In plants, at least 17 families of PR proteins have been identified and characterized. The families are numbered in the order in which they were discovered (Van Loon et al., 2006).

The first PR (PR-1) protein family was identified in the early 1980s (Antoniw et al., 1980). PR-1 proteins are produced most abundantly upon pathogen attack in well-studied plant species (Alexander et al., 1993; Buchel and Linthorst, 1999). For example in tobacco, PR-1 proteins can account for 1% of the total leaf proteins in TMV-infected tissue (Datta and Muthukrishnan, 1999). PR-1 proteins are conserved among diverse plant species (Van Loon, 1999). All PR-1-like proteins contain a signal peptide of 24 to 25 amino acid resides at the N-terminus and a conserved three-dimensional structure called 'PR-1 fold' that consists of four α-helices and one four-strand β-sheet represented by the tomato PR-1 protein *P1a* (Van Loon and Gerritsen, 1989). The hydrophobic signal sequence existing in the primary translation products is cleaved off upon entry in the endoplasmic reticulum (Van Loon and Gerritsen, 1989). The 'PR-1 fold' is fundamental for the high stability of PR-1 proteins and their insen-sitivity to several proteases. PR-1 proteins also contain a highly conserved six-cysteine motif, critical for disulfide bond formation (Fernandez et al., 1997).

In plants, PR-1 genes comprise a multi-gene family. However, only a few members from this family showed inducible expression and possessed inhibitory activity against pathogens (Li et al., 2011). *Arabidopsis* and rice contain 22 and 39 PR-1 type genes, but only 1 and 2, respectively have been found to be inducible by pathogen or insect attacks (Van Loon et al., 2006). In a previous study, none of the three tested PR-1 genes in apple (*Malus domestica* B.) showed inducible expression in response to inoculation with pathogen or by treatment with SAR elicitors (Bonasera et al., 2006).

Grape is the most economically important perennial fruit crop worldwide, but cultivated grapevine (*Vitis vinifera*) is susceptible to many pathogens, such as downy mildew, powdery mildew and anthracnose. Anthracnose, caused by the fungus *Elsinoe ampelina* (de Bary) Shear, is a very destructive wet weather fungal disease of grapes (Kang et al., 2008). Although, control of anthracnose on grapevine is currently achieved by the widespread of chemicals, however, the development of resistant grape cultivars against anthracnose will reduce labor costs associated with chemical spraying and the undesirable impacts of chemicals on environment. Introducing disease resistance gene(s) from resistant species into susceptible species is an important way to develop novel cultivars resistant to pathogen attacks (Bisson et al., 2002).

In previous research, we constructed a subtractive suppression hybridization (SSH) cDNA-library to identify and verify genes that were differentially expressed during a pathogen response of *Vitis quinquangularis* 'Shang-24' to *E. ampelina* (Wang, 2011). In this SSH cDNA-library, we obtained a PR-1 gene cDNA sequence with complete open reading frame (ORF). In *V. vinifera* genome 21 PR-1 genes have been identified (Li et al., 2011). However, the expression patterns of grape PR-1 genes in response to pathogen attack are still unknown, inhibiting our understanding of the roles of PR-1 proteins in grape disease resistance. This research reported herein, detailed the results of the sequence characterization and expression patterns of the PR-1 gene from Chinese wild *V. quinquangularis* clone 'shang-24', with the hope that this novel pathogen-responsive PR-1 gene will benefit future research in the improvement of grape pathogen resistance.

MATERIALS AND METHODS

Highly *E. ampelina*-resistant *V. quinquangularis* clone 'Shang-24' was maintained in grape germplasm resources orchard of Northwest A&F University, Yangling, China.

E. ampelina inoculation and SA, Eth and JA Treatments

When shoots of vines were 25 to 35 cm in length, the third to fifth fully expanded young grapevine leaves beneath the apex were selected for *E. ampelina* inoculation and signaling molecule treatments. *E. ampelina* was originally collected from the infected leaves of *V. vinifera* L. 'Red Globe'. Spores were suspended in sterile distilled water and the density was adjusted to 2×10^6 spores/ml. At 7: 00 am on June 25th, 2008, healthy leaves of 'Shang-24' were inoculated with 0.5 ml of spore suspension of *E. ampelina*. Leaves sprayed with sterile water were used as negative control. Immediately after inoculation, the inoculated leaves were covered with plastic bags for 12 h to maintain humidity. Inoculation was repeated three times on three independent plants and leaves were collected at 0, 4, 8, 12, 24, 48, 72 and 120 h post inoculation (hpi), and immediately frozen in liquid nitrogen for further study.

To test if PR-1 expression is induced by plant defense signaling molecules, 100 μM SA (Wang and Li, 2006), 0.5 g/L Eth (Belhadj et al., 2008) and 50 μM MeJA (Repka et al., 2004) were sprayed on selected young leaves of 'Shang-24' under normal field conditions. Leaves sprayed with sterile water were used as negative control. Treatments were repeated three times on three independent plants and leaves were selected at 0, 0.5, 1, 3, 6, 12, 24 and 48 h post treatment (hpt). Samples were immediately frozen in liquid nitrogen for further study.

Total RNA extraction and purification

Total RNA of grape was extracted using improved SDS/phenol method (Zhang et al., 2003). Contaminated DNA was removed by DNase I (Promega, USA). Concentration of total RNA was detected by measuring UV absorbance at 260 nm. RNA purity was checked by determining the A_{260}/A_{280} ration, and RNA integrity was examined by 1% agarose gel electrophoresis.

Gene identification and sequence analysis

The positive clones in the SSH library were randomly selected and

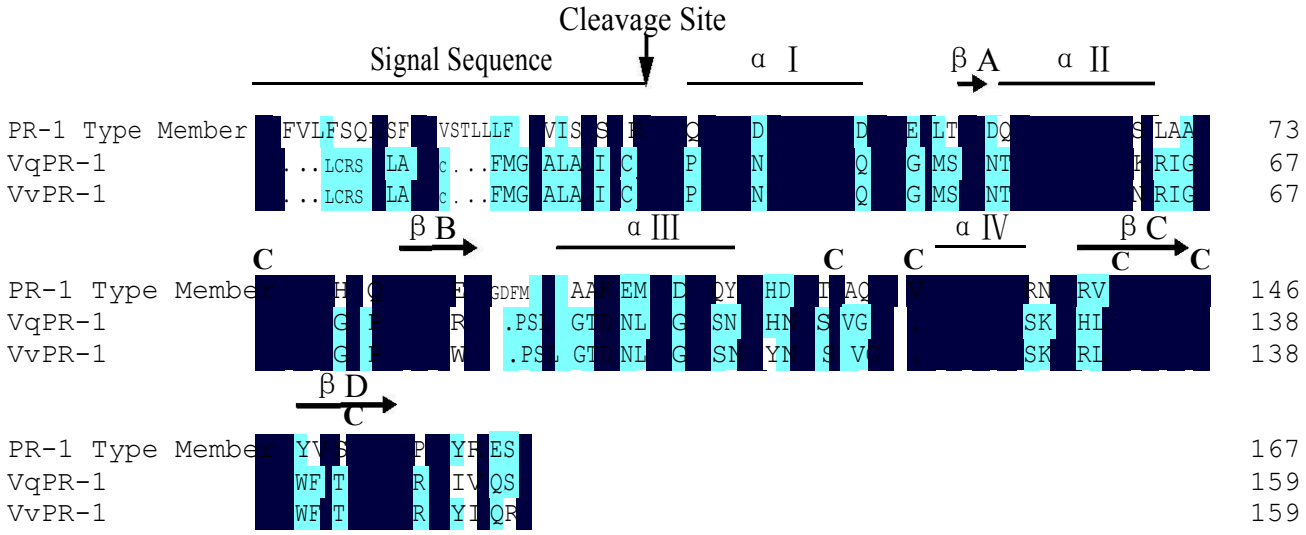

Figure 1. Alignment of the deduced amino acid sequences of *VqPR-1* gene, the type member *PR-1a* from tobacco (GenBank: CAA29392) and a *VvPR-1* gene from *V. vinifera* (GenBank: XP_002273788). The cleavage site between the signal peptide and mature protein is indicated by the arrowhead. The positions of six conserved cysteine residues, the four helices α(Ⅰ-Ⅳ), and the four parallel strands β-sheet (A-D) are marked.

sequenced. Sequences were compared to the UniProt database (http://www.uniprot.org/) as well as NCBI database using BLAST (http://www.ncbi.nlm.nih.gov/blast). Multiple protein alignment was performed with ClustalX 1.81 (Thompson et al., 1997).

Phylogenetic analysis

Phylogenetic tree was constructed with MEGA 4.0 (Tamura et al., 2007) by neighbor-joining (NJ) method and the bootstrap test was carried out with 1000 replicates. Pair-wise deletion opinion and Jones, Taylor, and Thornton (JTT) model for amino acid sequences were used.

Quantitative real-time analysis

First-strand cDNA was synthesized from 1 μg of DNase-treated total RNA using PrimeScript™ RTase (TaKaRa Biotechnology, Dalian, China). qRT–PCR was conducted using SYBR green (Takara Biotechnology) on an IQ5 real-time PCR machine (Bio-Rad, Hercules, CA, USA). Each reaction was done in triplicates with a reaction volume of 25 μL. The 25 μL PCR reaction contained 12.5 μL of SYBR® Premix Ex *Taq* TM II (2×), 1 μL of PCR Forward Primer (10 μM), 1 μL of PCR Reverse Primer (10 μM), 2 μL of 10 × diluted cDNA, and 8.5 μL of ddH_2O. Cycling parameters were 95°C for 30 s, 40 cycles of 95°C for 5 s, and 60°C for 30 s. To analyze the quality of the dissociation curves, the following program was added after 40 PCR cycles: 95°C for 15 s, followed by a constant increase from 60 to 95°C. Grapevine *Actin1* (GenBank accession no. AY680701) was amplified as internal control. Each relative expression level was analyzed with IQ5 software using the normalized-expression method.

The gene specific primer pairs used for qRT-PCR were as follows: PR-1, F: 5'- GCA ATC TAG TGC ATT CAG GTG G -3', and R: 5'- GCT CCA AAC AAC TTG AGT ATA G -3'; *Actin1*, F: 5'- GAT TCT GGT GAT GGT GTG AGT- 3', and R: 5'- GAC AAT TTC CCG TTC AGC AGT - 3'.

Semi-quantitative RT-PCR analysis

Organs of leaves, stems, tendrils, flowers (5 days after flowering) and pericarps (50 days after flowering) were collected from *V. quinquangularis* clone 'Shang-24' and used for organ-specific expression analysis by semi-quantitative RT-PCR technique. Grapevine *Actin1* gene (GenBank accession no. AY680701) was used as the control to standardize the expression of PR-1 gene. Semi- quantitative RT-PCR was performed at 94°C for 3 min, 30 cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 30 s and 72°C for 10 min. The gene specific primer pairs used for semi-quantitative RT-PCR were same with the primers used for qRT-PCR.

RESULTS

Identification and sequence analysis of *VqPR-1*

From the SSH library, a PR-1 gene cDNA sequence was obtained and designated as *VqPR-1* (GenBank accession no. JN256202). The length of cDNA sequence of *VqPR-1* was 529 bp, encoding a predicted polypeptide of 159 amino acids. An alignment of the peptide sequence of *VqPR-1* from 'Shang-24', a PR-1 protein from 'Pinot Noir' (*V. vinifera*; XP_002273788) and the PR-1 type member (tobacco *PR-1a*) (GenBank accession no. CAA29392) is shown in Figure 1. From the alignment, it appears that *VqPR-1*, the PR-1 from *V. vinifera* (XP_002273788) and PR-1 type member (tobacco *PR-1a*) are highly identical in sequence and they all contain the requisite signal sequence at the N-terminus, four α-helices, one four-strand β-sheet and a highly conserved six-cysteine motif critical for disulfide bond formation, present in the PR-1 family of proteins (Van Loon and Van Strien, 1999). We

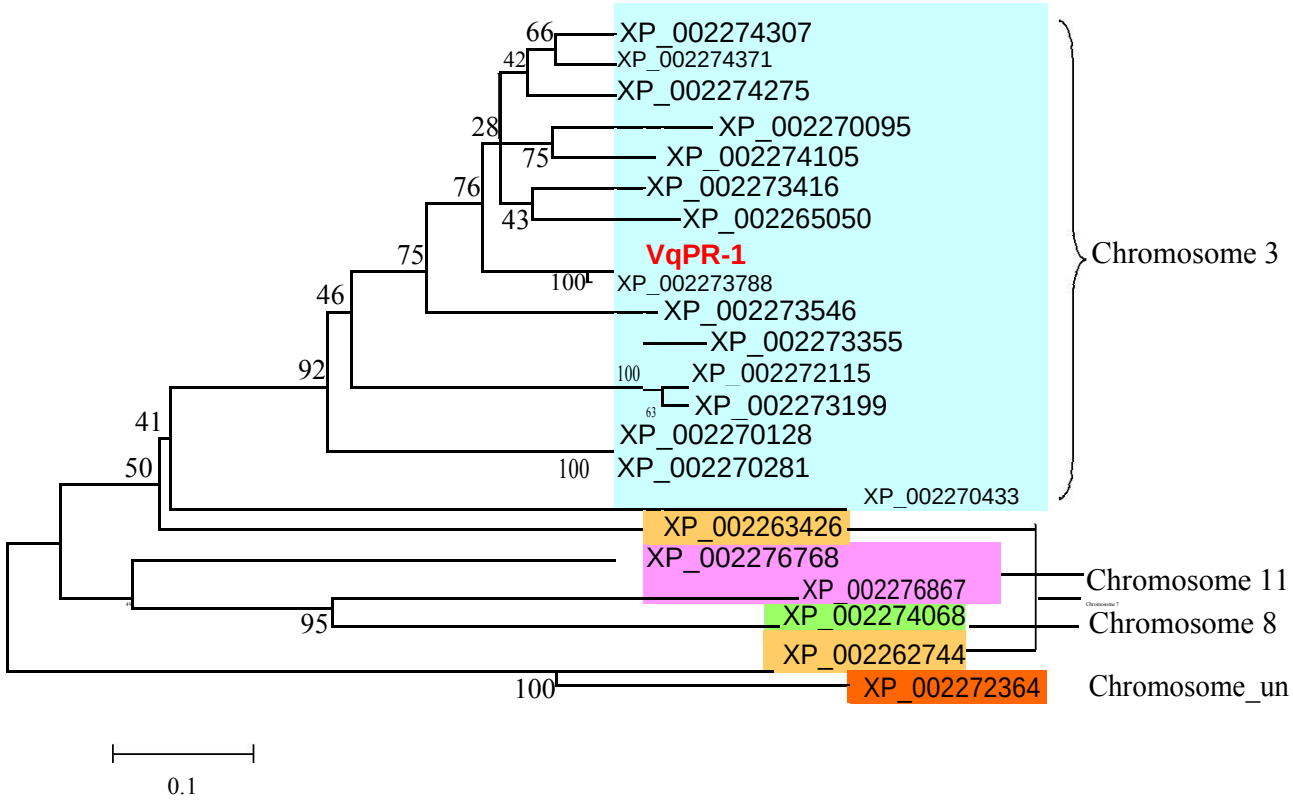

Figure 2. Phylogenetic relationship of *VqPR-1* protein and 21 *VvPR-1* proteins from *V. vinifera.* The 21 *VvPR-1* proteins were named with their GenBank accession numbers. The unrooted NJ tree was generated with MEGA 4.0 program. Bootstrap values from 1000 replicates are indicated at each branch. The five major groups representing the chromosome which they are located at are indicated.

could also conclude that *VqPR-1* cDNA sequence contains the complete open reading frame (ORF).

Phylogenetic analysis of PR-1 genes

In order to compare the *VqPR-1* gene from *V. quinquangularis* clone 'Shang-24' and *VvPR-1* genes from *V. vinifera* and explore their evolutionary relationships, a phylogenetic tree was constructed from the alignment of the full-length PR-1 proteins. The PR-1 genes located at the same chromosome (as determined from the grape genome sequence; Li et al. 2011) tend to be clustered together (Figure 2). This is especially apparent for those PR-1 genes located on chromosome 3, with all 15 genes, representing over 70% members of the PR-1 family, being grouped into the same cluster. From this phylogenetic tree, it appears that the *VqPR-1* protein from *V. quinquangularis* clone 'Shang-24' is highly similar in sequence to the protein acc no. XP_002273788 located within the chromosome 3 cluster. Amino acid alignment analysis found that *VqPR-1* protein had a high sequence identity of 95.6% to *VvPR-1* protein accession no. XP_002273788 from *V. vinifera* 'Pinot Noir' (Figure 1).

Expression of *VqPR-1* was induced rapidly by *E. ampelina* and plant defense signaling molecules

To determine whether *VqPR-1* responds to infection by *E. ampelina*, qRT-PCR was conducted (Figure 3), and results of qRT-PCR showed that expression of *VqPR-1* was induced rapidly by *E. ampelina* in Chinese wild *V. quinquangularis* clone 'Shang-24'. Expression level of *VqPR-1* went up rapidly and peaked at 8 hpi, undergoing an approximately 10 fold increase. Then it decreased to its lowest level at 24 hpi, after which it increased smoothly until 120 hpi.

To test whether *VqPR-1* was induced by defense signaling molecules, the selected leaves of Chinese wild *V. quinquangularis* clone 'Shang-24' were treated with SA, MeJA and Eth. According to Figure 4, three plant defense signaling molecules all induced the expression of *VqPR-1* gene, although the induction of MeJA was not obvious. After the treatment of three signaling molecules, expression levels of *VqPR-1* all grew steadily and reached the first peak at about 0.5 hpt, but after that, they declined gradually and reached the lowest levels at around 3 hpt. Then, the expression levels surged to their second peak at 12 hpt, followed by a dramatic drop to be

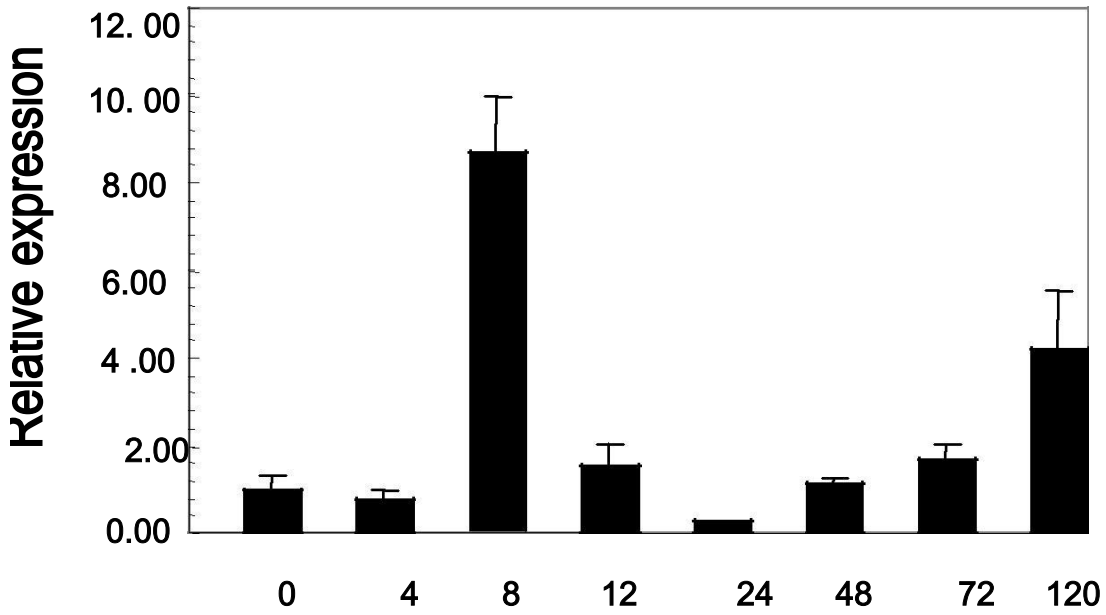

Figure 3. Expression profiles of *VqPR-1* at different time points after inoculation with *E. ampelina* in *V. quinquangularis* clone 'Shang-24'. Grapevine *Actin1* gene was used as internal control for qRT-PCR and the relative expressions indicate expression level in treated leaves compared with the negative control, which was set to 1. Mean values and SDs were obtained from three technical and three biological replicates.

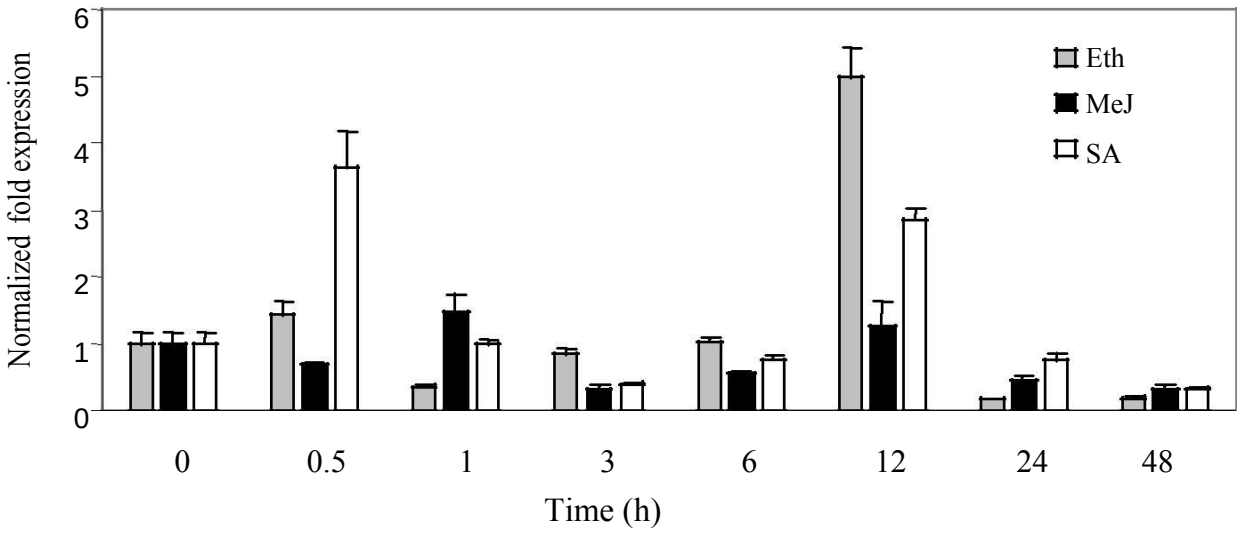

Figure 4. Expression profiles of *VqPR-1* induced by different plant defense signaling molecules in *V. quinquangularis* clone 'Shang-24'. Grapevine *Actin1* gene was used as internal control for qRT-PCR and fold expressions indicate expression level in treated leaves compared with the negative control, which was set to 1. Mean values and SDs were obtained from three technical and three biological replicates.

lower than half of the basal expression level at 48 hpt.

***VqPR-1* gene was differentially expressed in various organs.**

To test whether *VqPR-1* was constitutively expressed in various organs, the expression profile of *VqPR-1* gene in different grape organs was analyzed by semi-quantitative RT-PCR (Figure 5). Grapevine *Actin1* (GenBank accession no. AY680701), which was expected to show a constitutive expression pattern, was used as the control to standardize the expression of the *VqPR-1* gene. There was a significant difference on the *VqPR-1* gene transcription

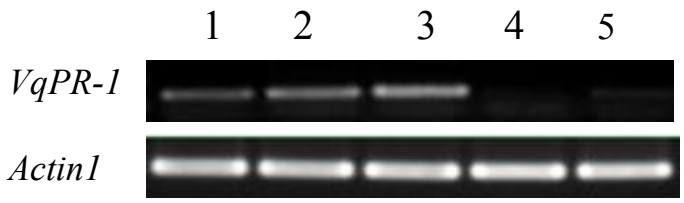

Figure 5. Semi-quantitative PCR analysis of *VqPR-1* mRNA in different organs from *V. quinquangularis* clone 'Shang-24'. Amplification of grapevine *Actin1* cDNA was used as control to standardize the expression of the *VqPR-1* gene. Lane 1: leaves, lane 2: Stems, lane 3: Flowers, lane 4: Pericarps and lane 5: Tendrils.

level in different organs. The high level of *VqPR-1* mRNA was found in flowers, leaves and stems, and the maxi-mum was observed in flowers. By contrast, the expression levels of *VqPR-1* were too low as detected in tendrils and pericarps.

DISCUSSION

In this study, we isolated a PR-1 gene from *V. quinquangularis* clone 'Shang-24'. Amino acid sequence analysis and sequence alignment with the type member (tobacco *PR-1a*) showed that *VqPR-1* protein contained the requisite conserved features of PR-1 proteins. Therefore, we concluded that the gene isolated here is of the PR-1 type. Furthermore, our phylogenetic tree revealed that grape PR-1 genes located on the same chromosome tended to be clustered together. *VqPR-1* was highly identical to the *VvPR-1* protein accession no. XP_002273788 (95.6% similarity) located on the chromosome 3 of *V. vinifera* 'Pinot Noir'. In *V. vinifera* genome, 21 unique PR-1 genes were identified, most of which are present in clusters and a cluster on chromosome 3 account for 70% of all PR-1 genes (Li et al., 2011). This phenomenon was also found in rice and *Arabidopsis* (Van Loon et al., 2006). PR-1 proteins from taxonomically diverse plant species seem to share not only highly conserved structures but also a similar pattern of gene organization within the genome (Li et al., 2011). Genes that participated in extracellular communication, including host immune response, often possessed a clustering organization (Chuang and Li, 2004). PR-1 genes, like other genes with a clustering organization, may have an essential role in plant immune response or perhaps in developmental regulation.

The ability of plants to protect themselves from various biotic and abiotic stresses depends on the number of proteins which are up- and down- regulated. Through SSH, we obtained 1024 sequences that induced by the *E. ampelina* from an anthracnose-resistant Chinese wild *V. quinquangularis* clone 'shang-24' (Wang, 2011). However, among the 1024 sequences, we only identified one PR-1 gene sequence. *V. vinifera* genome contains 21 unique PR-1 genes (Li et al., 2011). In our cDNA library, we did not identify the other 20 PR-1 genes. Maybe this is because the sequenced clones were randomly selected from the SSH library, so we did not select the clones that contained the other 20 PR-1 genes. However, there is another possibility that only few members of PR-1 family are inducible by pathogens. Similar findings were also observed in other well-studied plant species. Only 1 out of 22 *Arabidopsis* PR-1-type genes and 2 out of 39 rice PR-1-type genes have been found to be inducible by pathogen or insect attacks, whereas many other PR-1 genes are expressed constitutively in roots or floral tissues (Van Loon et al., 2006). As a result, it is likely that the *VqPR-1* gene identified in present study is a rare pathogen-responsive PR-1 gene. The expression profile of *VqPR-1* following inoculation of grape young leaves with *E. ampelina* also demonstrated that *VqPR-1* was induced by the pathogens attack. After inoculation, the expression level of *VqPR-1* surged rapidly. However, we observed a strange phenomenon that the expression level declined dramatically after 8 hpi. At 24 hpi, the expression level was so low that we nearly could not detect it by qRT-PCR. A possible explanation for this is that pathogens suppressed plant defenses. Although, plants have evolved a sophisticated network of defense mechanisms to counter microbial infections, however the plant defense network is not infallible. Successful pathogens have evolved strategies to suppress plant defense responses (Abramovitch and Martin, 2004). Maybe the dramatic decline of *VqPR-1* gene expression was the result of *E. ampelina's* suppression of grape defenses.

Plant defense responses to microbial attack are regulated through a complex network of signaling pathways that involve three signaling molecules: SA, JA, and ET (Glazebrook, 2005). These signaling molecules are involved in two major pathogen defense signaling pathways: an SA-dependent pathway and an SA-independent pathway that involves JA and ET (Kunkel and Brooks, 2002). These pathways do not function independently, but rather influence each other through a complex network of regulatory interactions. Several PR genes, the expression of which is dependent on SA, are commonly used as reporters of SA-dependent defense. It has been demon-strated that not only SA, but also JA and ET could induce the production of PR proteins (Kunkel and Brooks, 2002). This study showed that both SA and Eth significantly induced the expression of *VqPR-1*, however, the induction of MeJA was not obvious. The result indicates that the expression *VqPR-1* was highly likely to be regu-lated by SA and Eth for the potential positive interactions between ET and SA pathways. The possible reason for MeJA's weaker induction is that JA pathway may not play a major role in the induction of *VqPR-1* expression, or it may even not be involved in the regulation of *VqPR-1* expression.

Moreover, it is worth noting that treated with signaling molecules, the expression level of *VqPR-1* experienced a

decline in some periods. For example, between 24 and 48 hpt, the expression level was much lower than the basal expression level. The expression of *VqPR-1* seemed to be suppressed. The underlying suppression mechanisms wait to be determined, but it reflects the complexity of plant defense signaling pathways: defense signaling molecules could not only induce, but also suppress the expression of defense genes. In addition, in order to test whether *VqPR-1* is constitutively expressed in all organs, the translational level of *VqPR-1* in various organs was investigated under normal developmental condition. The highest expression of *VqPR-1* was observed in flowers, stems and leaves. Previous findings also showed that many defense-related proteins are present constitutively in floral tissues (Van Loon et al., 2006). However, compared with other organs, there were no detectable *VqPR-1* transcripts in pericarps and tendrils. Hence, defense pattern may be speculated as below: *VqPR-1* maintained at a relative low expression level in some plant organs, such as flowers, stems and leaves under normal developmental process, but its expression level surged rapidly to resist pathogen invasion as soon as it is subjected to pathogen attacks.

Conclusion

Most members of PR-1 family are constitutively expressed and not inducible by pathogen attacks, whereas *VqPR-1* was highly likely to be a rare pathogen-responsive PR-1 gene. *E. ampelina* inoculation could trigger a rapid induction of *VqPR-1.* However, we also observed that the expression of *VqPR-1* was suppressed at some point in time during the inoculation, which may be the result of pathogen's suppression of plant defenses. *VqPR-1* can be induced by SA and Eth, which suggests that *VqPR-1* may interact with transduction pathways of SA and ET. The weaker induction of MeJA compared with SA and Eth suggests JA pathway may not play an essential role in the activation of *VqPR-1* gene. The different expression patterns of *VqPR-1* in various organs suggest that *VqPR-1* functions mainly in floral tissues, stems, and leaves. Further studies on the analysis of *VqPR-1* functions using transgenic plants will reveal the exact roles and functions of *VqPR-1* protein involved in the grape defense systems.

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