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Expression of a GDP-L-galactose phosphorylase-like gene in a Chinese wild *Vitis* species induces responses to *Erysiphe necator* and defense signaling molecules

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ABSTRACT. Using rapid amplification of cDNA ends, a full-length cDNA sequence of a GDP-L-galactose phosphorylase-like gene was isolated from leaves infected by *Erysiphe necator* in the Chinese wild (*Vitis pseudoreticulata*) clone, 'Baihe-35-1', an *E. necator*-resistant genotype. The full-length cDNA, designated as *VpVTC*, comprised 1943 bp and putatively encodes a 453-amino acid polypeptide containing an HIT motif. The deduced amino acid sequence showed high similarity with that of *VTC* genes from other plants. The expression of *VpVTC*, determined by reverse transcriptase-polymerase chain reaction, was induced by *E. necator* and defense signaling molecules, including salicylic acid, methyl jasmonate, and ethephon, in 'Baihe-35-1', the *V. quinquangularis* genotype 'Shang-24', and the *E. necator*-susceptible

V. pseudoreticulata genotype, 'Hunan-1'. Transcript levels of *VpVTC* correlated well with the degree of disease resistance in the 3 genotypes. Maximum induction of *VpVTC* by *E. necator* (>7-fold at 96 h post-inoculation) occurred in 'Baihe-35-1', which also showed the fastest response to signaling molecules. Upregulating the expression of *VpVTC* in 'Baihe-35-1' resulted in a gradual increase in the ascorbic acid concentration of leaves inoculated with *E. necator*. Furthermore, *VpVTC* was expressed in leaves, stems, inflorescence, tendrils, and fruit at all developmental stages, with the highest level occurring in fruit 35 days after flowering.

Key words: *Vitis*; GDP-L-galactose phosphorylase; *Erysiphe necator*; Salicylic acid; Methyl jasmonate; Ethephon

INTRODUCTION

Vitamin C (L-ascorbic acid, AsA) is well known as an important antioxidant that acts as an enzyme cofactor and redox status indicator in both animals and plants. It has many important functions in plants. For example, it is involved in processes such as reactive oxygen species (ROS) detoxification (Padh, 1990), cell division and growth (Horemans et al., 2000), and the synthesis of hydroxy-proline (Arrigoni et al., 1977) and plant hormones (Arrigoni and De Tullio, 2002). Several possible biosynthetic pathways for ascorbate have been described, including the L-galactose, L-glucose pathway, D-galacturonic acid, and the myo-inositol pathways.

The L-galactose pathway is the major biosynthetic route for the production of L-ascorbate in higher plants, a vital antioxidant. The last unknown enzyme involved in this pathway has recently been identified as a GDP-L-galactose phosphorylase (GGP). This enzyme catalyzes the first committed step in the synthesis of L-ascorbate and its cofactor (Linster and Clarke, 2008). At least 2 genes, *VTC2* and *VTC5*, were found to encode GGP in *Arabidopsis thaliana* (Linster et al., 2007). Transient expression of a homologous gene (GenBank accession No. At4g26850) from *Actinidia chinensis* in tobacco leaves resulted in increases in the leaf ascorbate content and GDP-L-galactose-D-mannose-1-phosphate guanyltransferase activity (Laing et al., 2007). The *VTC2* gene was expressed throughout all developmental stages of *A. thaliana*, but at higher levels in green tissues than in the root (Müller-Moulé, 2008). A loss-of-function double mutant for *VTC2* and *VTC5* showed growth arrest immediately upon germination and the cotyledons subsequently bleached if not supplemented with ascorbate or L-galactose. Together, these results show that the 2 genes encoding GGP in *A. thaliana* are required for ascorbate biosynthesis and seedling viability, and that the L-galactose pathway is the only significant source of L-ascorbate in *A. thaliana* seedlings (Dowdle et al., 2007).

In recent years, the role of GGP in plant disease resistance was confirmed by mutant analysis. Two AsA-deficient mutants, *VTC1* and *VTC2*, were much more resistant to virulent *Pseudomonas syringae* pv. *maculicola* ES4326 and to the downy mildew pathogen *Peronospora parasitica* pv. *Noco* compared to the wild-type (Barth et al., 2004). Both mutations, *VTC1* and *VTC2*, conferred the ability to restrict *P. syringae* pv. *tomato* proliferation by 15- and 13-fold, respectively, over wild-type levels (Pavet et al., 2005). Enhanced resistance to *P. syringae* revealed that *VTC1* and *VTC2* mutations limit bacterial proliferation and cell death

expansion (Pavet et al., 2005). The enhanced pathogen resistance correlated with higher transcript and protein levels of PR1 and PR5, increased levels of salicylic acid (SA), and premature senescence (Barth et al., 2004). Low AsA concentrations in *VTC1* activated expression of SA-regulated genes; a response found to be dependent on the natriuretic peptide receptor A/guanylate cyclase A (Brosché and Kangasjärvi, 2012). However, the enhanced resistance of *VTC1* was also found to be prevented by a 0.5-mM AsA treatment, which also resulted in similar phenotypes between *VTC1* and wild-type plants (Wang et al., 2011a). Moreover, Davey et al. (2007) showed that AsA content increased after pathogen inoculation. For example, after infection by *Botrytis cinerea*, the fruit was able to mount a rapid and efficient defense response and maintained a generally higher AsA content.

Powdery mildew (PM) caused by *Erysiphe necator* Schw. is, economically, the most important fungal disease of grapes. *E. necator* significantly reduces vine growth, yield, fruit quality, and winter hardiness, and therefore represents a serious threat to fruit production in many vineyards throughout the world. Although several viticulture practices, such as the use of fungicides, are able to manage the diseases' impact, their application is expensive and laborious. Chinese wild *Vitis* species are valuable sources of resistance to *E. necator* (Wang et al., 1995). Understanding the hosts' defense mechanism and the identification of resistant genes should provide valuable information and resources for molecular protocols in breeding resistant cultivars.

In our previous study, we screened for genes induced in the leaves of the *Vitis pseudoreticulata* W.T. Wang genotype, 'Baihe-35-1', after infection with *E. necator* (Wang et al., 2011b). Using differential display reverse transcriptase-polymerase chain reaction (RT-PCR), a cDNA fragment of a GGP-like gene was obtained. In this study, we cloned the full-length GGP-like cDNA using rapid amplification of cDNA ends (RACE), which was then characterized. Furthermore, we analyzed its expression by semi-quantitative RT-PCR in various organs in 'Baihe-35-1' and assessed the impact of *E. necator* infection and defense signaling molecules on transcript levels by qRT-PCR. Finally, we assayed the AsA concentration in different grapevine genotypes infected by *E. necator*.

MATERIAL AND METHODS

Plant and pathogen materials

Three grapevine genotypes were tested in this study, including 2 resistant genotypes and 1 susceptible genotype. The *E. necator*-resistant grapevine genotypes were *V. pseudoreticulata* genotype 'Baihe-35-1' and *V. quinquangularis* genotype 'Shang-24'. The susceptible genotype was *V. pseudoreticulata* genotype 'Hunan-1'. These genotypes were maintained in the grape germplasm resources orchard of Northwest A&F University, Yangling, China (34°200'N, 108°240'E). When shoots of the vines were 30-40 cm in length, the third to sixth fully expanded young leaves below the apex were selected for treatments (Li et al., 2010a). *E. necator* was collected from the highly susceptible *V. adstricta* Hance genotype 'Taishan-2' in the grape germplasm resource orchard, Northwest A&F University.

Observation of mycelial growth stages on grape leaves infected by *E. necator*

Sampling, fixation, and clearing of leaf tissues for microscopy were performed as

described in Vanacker et al. (2000). To stain fungal structures for micrography, a drop of aniline blue [0.1% (v/v) in lactoglycerol] was pipetted onto leaf surfaces immediately before they were photographed.

Biotic and signaling molecule treatments

Maintenance and inoculation of grapevine with *E. necator* were performed as described previously (Zhou et al., 2007; Wang et al., 2011b). Leaves sprayed with sterile water were used as negative controls. At each inoculation, leaves were sampled at 0, 6, 12, 24, 48, 72, 96, and 120 h post-inoculation (hpi), immediately frozen in liquid nitrogen, and stored at -80°C. Selection and treatment of grapevine leaves with defense signaling molecules [SA, methyl jasmonate (MeJA), and ethephon (Eth)] were performed as described in Li et al., 2010a. The treated grapevine leaves were sampled at 0, 0.5, 1, 3, 6, and 12 h, immediately frozen in liquid nitrogen, and stored at -80°C.

Total RNA extraction

Total RNA was extracted using an improved SDS/phenol protocol (Zhang et al., 2003). Residual DNA was removed by DNase I (Promega, Madison, WI, USA). RNA purity was checked by determining the A_{260}/A_{280} ratio, and RNA integrity was examined by electrophoresis on 1% agarose gel. The concentration of total RNA was measured with a spectrophotometer (V-550, JASCO, Japan) at 260 nm.

Full-length cloning of *VpVTC* and sequence analysis

In order to obtain the full-length cDNA of *VpVTC*, RACE-PCR amplification was carried out. A gene-specific primer (GSP: 5'-TCT CCC ATA ACC ATT GAT TTC GCC GCA C-3') was designed based on the partial sequence of the differential cDNA fragment *VpVTC*. Total RNA was isolated from 'Baihe-35-1' leaves infected with *E. necator*, which were harvested at 120 hpi, as described above. RACE was performed according to the manufacturer instructions (BD SMART™ RACE cDNA Amplification Kit; Clontech, Palo Alto, CA, USA). The resulting PCR products were transformed into *Escherichia coli* strain DH5α. A positive candidate clone was sequenced at TaKaRa Biotechnology. Sequence multiple alignment was performed using the DNAMAN software.

VTC expression analysis by qRT-PCR in different grapevine genotypes

qRT-PCR and data analysis were conducted as described in Li et al. (2010a). First-strand cDNA was synthesized from 1 µg DNase-treated total RNA using PrimeScript™ RTase (TaKaRa Biotechnology). qRT-PCR was conducted using SYBR green (TaKaRa Biotechnology) on an IQ5 real-time PCR machine (Bio-Rad, Hercules, CA, USA). Each reaction was performed in triplicate. The 25 µL PCR contained 12.5 µL 2X SYBR® Premix Ex *Taq*™ II, 1 µL 10 µM PCR forward primer, 1 µL 10 µM PCR reverse primer, 2 µL 10X diluted cDNA, and 8.5 µL ddH_2O. Cycling parameters were 95°C for 30 s, 40 cycles at 95°C for 5 s, and 60°C for 30 s. To analyze the quality of dissociation curves, the following program was added after 40 PCR cycles: 95°C for 15 s, followed by a constant increase in temperature from 60° to 95°C.

Grapevine *Actin1* (GenBank accession No. AY680701) was amplified as an internal control. The gene-specific primer pairs used for qRT-PCR were as follows: for *VpVTC*, F: 5'-GCC TAC AAG AAG GTG AGG GAC G-3', and R: 5'-GAG AGA GTC AAG GAA GGG CAC AG- 3', and for *Actin1*, F: 5'- GAT TCT GGT GAT GGT GTG AGT-3' and R: 5'-GAC AAT TTC CCG TTC AGC AGT-3'. Each relative expression level was analyzed with the IQ5 software using the Normalized Expression method. A one-side paired *t*-test was performed to assess significant differences between the negative control and the treatment, using SigmaPlot 11.0 (Ashburn, VA, USA).

VpVTC expression analysis by semi-quantitative RT-PCR in various organs and developmental stages of fruit

Total RNAs were extracted from the leaf, stem, tendril, inflorescence, and fruit of 'Baihe-35-1' at 20, 35, 50, 65, 80, and 95 days after flowering (daf). The specific primer sequences of *VpVTC* and the internal control were the same as those used for qRT-PCR. Each reaction was carried out in triplicate in a 20-μL reaction volume. The cycling program was 94°C for 3 min, followed by 25 cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 30 s, 72°C for 10 min. Eight microliters of each PCR product was examined on a 1.2% (w/v) agarose gel, then visualized and photographed under UV light after staining with ethidium bromide.

Assay of AsA

AsA was assayed according to methods described in Kampfenkel et al. (1995) and Li et al. (2009).

RESULTS

Gene cloning and sequence analysis

A 1943-bp product was obtained from the Chinese wild *V. pseudoreticulata* genotype 'Baihe-35-1', using the RACE technique with the GSP-specific primer. It contains a 1359-bp open reading frame, a 292-bp 5'- and a 292-bp 3'-untranslated region (Figure 1) and encodes a polypeptide of 453 amino acids containing an HIT motif (Figure 2), which was designated *VpVTC* (accession No. JN566043).

We aligned the deduced amino acid sequence of *VpVTC* with the sequences of other plant *VTCs* and constructed a phylogenetic tree (Figure 2). The deduced amino acid sequence of *VpVTC* was highly similar to those of other plant *VTCs*, which shared the same conserved HIT (histidine triad, His-Leu-His-Phe-Gen) motif (Figure 2). In addition, the phylogenetic tree indicated that *VpVTC* was closely related to *VTCs* of fruit trees, such as *Actinidia deliciosa* GGP, *Citrus unshiu VTC*, *Malus* x *domestica VTC2*, *Rosa*, *roxburghii VTC*, and *Malpighia glabra VTC* (Figure 2).

VTC expression is induced by *E. necator* infection

Microscopy observations of mycelial growth of *E. necator* on leaves of 'Baihe-35-1',

'Shang-24', and 'Hunan-1' at 120 hpi verified that the *V. pseudoreticulata* genotype 'Hunan-1' was more susceptible to *E. necator* than the *V. pseudoreticulata* genotype 'Baihe-35-1' and the *V. quinquangularis* clone 'Shang-24' (Figure 3A). To investigate whether expression of *VTC* varied among different grapevine genotypes infected by *E. necator*, we analyzed *VTC* transcript levels by qRT-PCR, which initially increased in the resistant genotypes 'Baihe-35-1' and 'Shang-24', and then ultimately decreased (Figure 3B). Maximal levels were observed in 'Baihe-35-1' at 96 hpi (over 8-fold). In the other resistant genotype, 'Shang-24', transcript levels reached their maximum at 72 hpi. In contrast to the resistant genotypes, *VTC* was not induced in the susceptible genotype 'Hunan-1' (Figure 3B).

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1     TCTCCCATAACCATTGATTTCGCCGCACGGCGGGCGTGGCGCTTTGCCTTCAGAAGGCGGTAGTCCCTCTGATCT
76    GCTCTTCCTCGCCGGCGGTGGTTCCAACGCTTTCCTCTGCTAGTTTAGGCTTATATTCTGTATAATATAGCTACT
151   GTCTTTAGGATTAGATCAACCAATCCGTATCGAACACTCGATCTCTCGCTTTAGCCATTTCTTTAGATCAATCAA
226   TCCGTATCGAACAATCGACCTCTCGCTTTAGCCATTTCTTTAGGTTGGTGTTTGAGTTCTTGAAAAA*ATG*AATAC
                                                                      M  N  T    3
301   GCTGAGGATTAAGAGGGTACCCACCGTGGTTTCGAATTACCAGAAGGAGGATTCAGATGATGGTGCTCGTCAGGT
       L  R  I  K  R  V  P  T  V  V  S  N  Y  Q  K  E  D  S  D  D  G  A  R  Q  V   28
376   TGGTGGTTGCGGCCGCAATTGCCTCAAGCAGTGCTGCATTCAAGGAGCAAAACTCCCTCTTTATGCCTACAAGAA
       G  G  C  G  R  N  C  L  K  Q  C  C  I  Q  G  A  K  L  P  L  Y  A  Y  K  K   53
451   GGTGAGGGACGTTGTTAACGAGAAAGCTTCGAGCGGTGATGAAAACAAAGAGCAGCCTGTGCCCTTCCTTGACTC
       V  R  D  V  V  N  E  K  A  S  S  G  D  E  N  K  E  Q  P  V  P  F  L  D  S   78
526   TCTCGTTCTTGGAGAGTGGGAGGATCGTATGCAGAAAGGGCTCTTTCGATATGATGTCACTGCTTGTGAAACCAA
       L  V  L  G  E  W  E  D  R  M  Q  K  G  L  F  R  Y  D  V  T  A  C  E  T  K  103
601   GGTGATTCCGGGTGAGTATGGGTTCATTGCCCAGCTGAATGAGGGCCGCCACCTGAAGAAGAGGCCCACTGAGTT
       V  I  P  G  E  Y  G  F  I  A  Q  L  N  E  G  R  H  L  K  K  R  P  T  E  F  128
676   CCGTGTGGATAAAGTCCTCCAGCCCTTTGATGGGAACAAATTCAACTTCACTAAAGTTGGGCAAGAGGAGGTGCT
       R  V  D  K  V  L  Q  P  F  D  G  N  K  F  N  F  T  K  V  G  Q  E  E  V  L  153
751   CTTCCAGTTTGAACCAAGCAATGATGAGGAACCTGAGTTCATCCCCGATGCTCCCATTGATGTCGAAAATTCTAC
       F  Q  F  E  P  S  N  D  E  E  P  E  F  I  P  D  A  P  I  D  V  E  N  S  T  178
826   AAGCGTTGTTGCCATCAATGTTAGTCCTATTGAATATGGGCATGTGCTTCTAATCCCAAGGATTTTCGAGTGCTT
       S  V  V  A  I  N  V  S  P  I  E  Y  G  H  V  L  L  I  P  R  I  F  E  C  L  203
901   GCCGCAGAGGATCGACCGTGAAAGCTTTTTGCTTGCCCTTGACATGGCCGTGGAAGCAGGAAATCCATATTTCCG
       P  Q  R  I  D  R  E  S  F  L  L  A  L  D  M  A  V  E  A  G  N  P  Y  F  R  228
976   GTTGGGTTACAACAGCTTGGGTGCATTTGCTACCATCAACCACCTTCACTTTCAGGCTTATTACTTGGCCACACC
       L  G  Y  N  S  L  G  A  F  A  T  I  N  H  L  H  F  Q  A  Y  Y  L  A  T  P  253
1051  CTTTCCCATTGAGAAGGCTCCAACTAGGAAAATAACCACTGCAGGAAATGGGGTGAAGATCTTTGAGCTGTTAAA
       F  P  I  E  K  A  P  T  R  K  I  T  T  A  G  N  G  V  K  I  F  E  L  L  K  278
1126  ATATCCTGTTAGAGGTCTTGTCTTTGAGGGTGGAGACACTCTGCAAGATTTAGCGAACACTGTGGCCGATTCCTG
       Y  P  V  R  G  L  V  F  E  G  G  D  T  L  Q  D  L  A  N  T  V  A  D  S  C  303
1201  CATTTGCCTTCAGGATAACAACATACCTTTCAATGTTCTCATTGCTGATGCTGGGAAACGTATCTTTCTCTTTGC
       I  C  L  Q  D  N  N  I  P  F  N  V  L  I  A  D  A  G  K  R  I  F  L  F  A  328
1276  ACAGTGTTATGCTGAGAAACAAGCTCTTGGGGAAGTGAATCAGGAGCTTCTGGACACCCAAGTGAACCCAGCTGT
       Q  C  Y  A  E  K  Q  A  L  G  E  V  N  Q  E  L  L  D  T  Q  V  N  P  A  V  353
1351  CTGGGAGGTTAGTGGACATATTGTGCTGAAAAGGAAGGAGGACTATGAGGGGGCATCTGAGGAGAATGCTTGGAG
       W  E  V  S  G  H  I  V  L  K  R  K  E  D  Y  E  G  A  S  E  E  N  A  W  R  378
1426  GCTTCTTGCTGAGGTCTCTCTCTCTGAAGAGAGGTTCCAAGAAGTGAATGCTCTTATCTTTGAAGCCATTGCCTG
       L  L  A  E  V  S  L  S  E  E  R  F  Q  E  V  N  A  L  I  F  E  A  I  A  C  403
1501  TGGAGATGATGAAAAAGGAAATCTCACCGAGGACATGATTGAGGAGCCAGATGTCACACCTCCATCTCATGAAGA
       G  D  D  E  K  G  N  L  T  E  D  M  I  E  E  P  D  V  T  P  P  S  H  E  D  428
1576  TGCAGGTGCCATCAACAATAGCTCCTACCCTGCTGCCATGGTGGCTGGAAAGCAAGAATGCCTAGTTCAGCAG*TA*
       A  G  A  I  N  N  S  S  Y  P  A  A  M  V  A  G  K  Q  E  C  L  V  Q  Q  *  453
1651  *A*GAGTTGGGCTTTTGAAGAACAGATGAATGGTGTTTGTGTTTCTGAGTCAGGTATCGGTGGTTTGGCATGGATGT
1726  TTCCGTGTCTCTGTGGTTTCTAGTAATTGCTTAAATAAAGCAAACTGGGTTTTGCACTGTTGTTGAAGTTGTGCC
1801  TGGTTTCTCTGCGTATTTGCTTTATGACCTATTGTTTATACGTGTCTCATGGGTCTGTAAAATTCTATGCTGTAC
1876  TTTGTGATGATTGAATAAATTTCTGTTCTTCGGTTTCTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
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Figure 1. Full-length cDNA sequence and deduced amino acid sequence of *VpVTC*. Nucleotide positions are given on the left side of the sequence in the 5'- to 3'-orientation. The start codon ATG and the stop codon TAA are italicized. The deduced amino acid sequence is shown beneath the nucleotide sequence and the amino acids are numbered on the right side of the sequence. The GSP primer and polyadenylation signal AATAAA are underlined sequences. The sequence has been deposited in GenBank (GenBank accession No. JN566043).

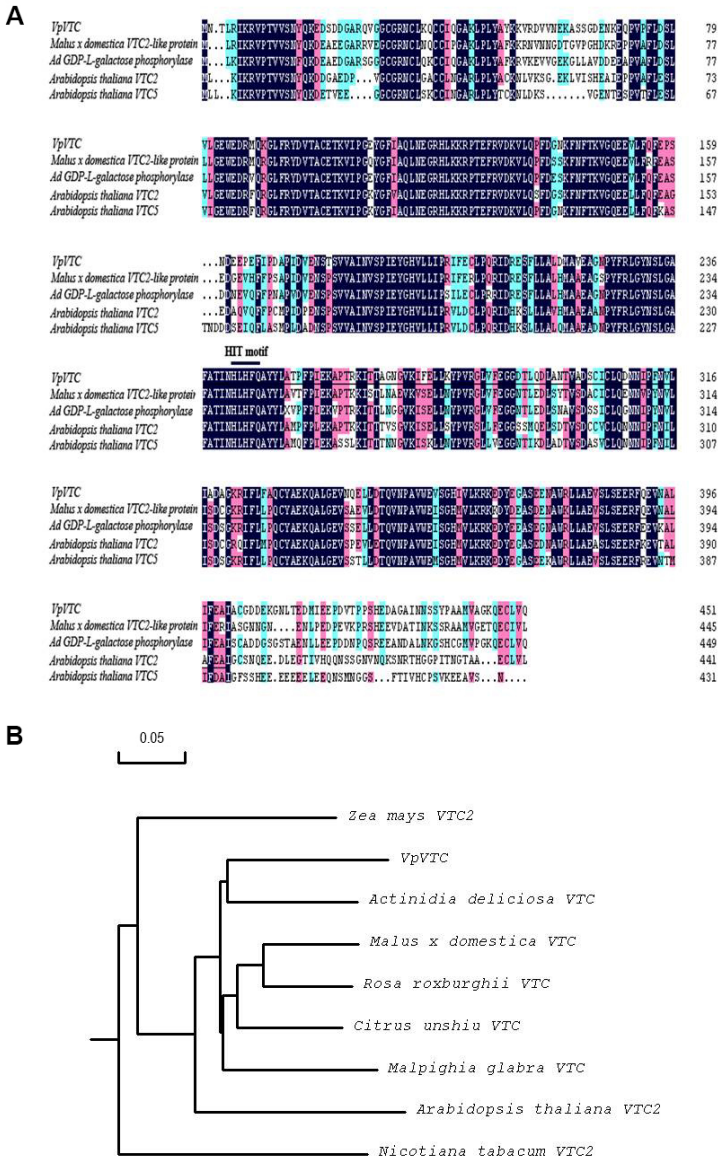

Figure 2. Multiple alignments (**A**) and the phylogenetic tree (**B**) of predicted amino acid sequences of *VpVTC* and reported *VTC* proteins in plants using the DNAMAN program. HIT motif is marked. Gaps to optimize alignments are designated by dots. The consensus amino acid identity among all organisms is black color. The amino acids are numbered on the right side of the sequence. The GenBank accession number of the *VTC* proteins are as follows: *Actinidia deliciosa* GDP-L-galactose phosphorylase (GenBank accession No. ADB85572), *Arabidopsis thaliana VTC2* (GenBank accession No. At4g26850) and *VTC5* (GenBank accession No. At5g55120), *Rosa roxburghii VTC* (GenBank accession No. ADM16545.1), *Malpighia glabra VTC* (GenBank accession No. ACG75920.1), *Zea mays VTC2* (GenBank accession No. NP_001150222.1), *Nicotiana tabacum VTC2* (GenBank accession No. ACD92981.1), *Citrus unshiu VTC* (GenBank accession No. ADV59925.1), and *Malus* x *domestica VTC2* (GenBank accession No. ACN88681).

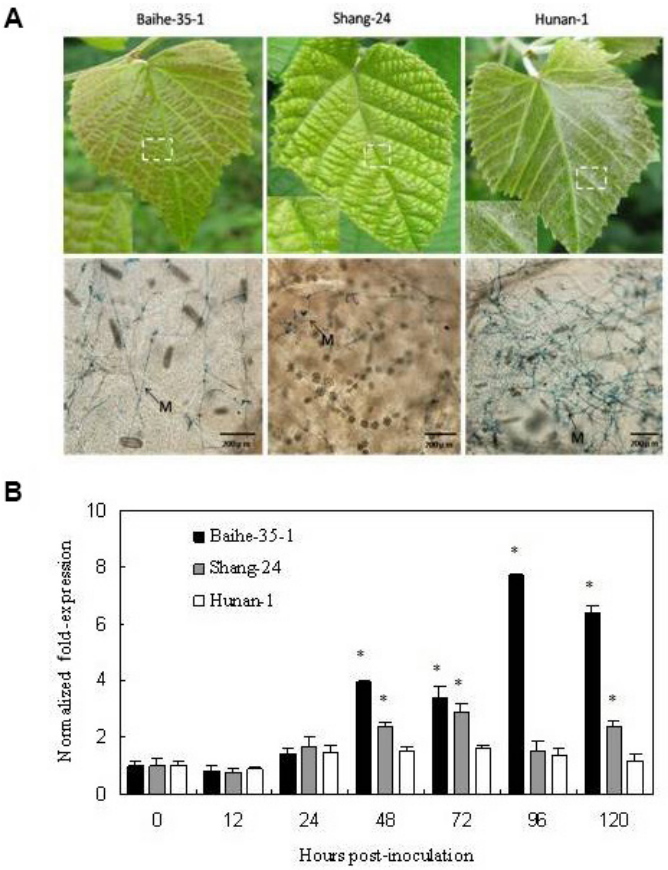

Figure 3. A. Phenotypic characterization and mycelial growth states on leaves of the resistant genotype 'Baihe-35-1' and 'Shang-24', and the susceptible genotype 'Hunan-1' at 120 h post-inoculation (hpi) after artificial inoculation of *Erysiphe necator*. Mycelium was stained by aniline blue. M = mycelium. Scale bar = 200 µm. **B.** Expression profiles of *VTC* was induced by *E. necator* in 3 grapevine genotypes. The maximum induction reached more than 7-fold 96 hpi in the resistant genotype 'Baihe-35-1' from which they were originally isolated. Expression was also induced in another resistant genotype 'Shang-24' and in the susceptible genotype 'Hunan-1'. *Actin1* was used as internal control for qRT-PCR and fold-expressions indicate expression level in treated leaves of each genotype compared with the negative control, which was set to 1. Asterisks indicate a significant difference ($P < 0.05$) in *VTC* expression. Mean values and SDs were obtained from 3 technical and 3 biological replicates.

Expression of *VTC* is induced by SA, MeJA, and Eth

The plant defense response is regulated through a complex network of signaling pathways that involve SA, MeJA, and Eth. To determine whether *VTC* also responded to plant defense signaling molecules, we further analyzed expression of *VTC* in leaves of 3 grapevine genotypes that were treated with SA, MeJA, or Eth, by qRT-PCR. After all 3 treatments, transcript levels of *VTC* increased the fastest and most strongly in the genotype 'Baihe-35-1' (Figure 4). Induction occurred rapidly by SA and MeJA in 'Baihe-35-1' and reached the maximum at 1 and 0.5 h, respectively, then gradually decreased to their initial levels at 12 h. After treatment with Eth, the peak of *VTC*

expression in 'Baihe-35-1' appeared at 6 h, and sharply decreased to its initial level at 12 h. *VTC* was also induced by SA and Eth in 'Shang-24', and although the trends were similar, they were less pronounced than those observed in 'Baihe-35-1'. In contrast, *VTC* did not show any response to SA treatment, and seemed only weakly induced by exogenous MeJA and Eth in 'Hunan-1'.

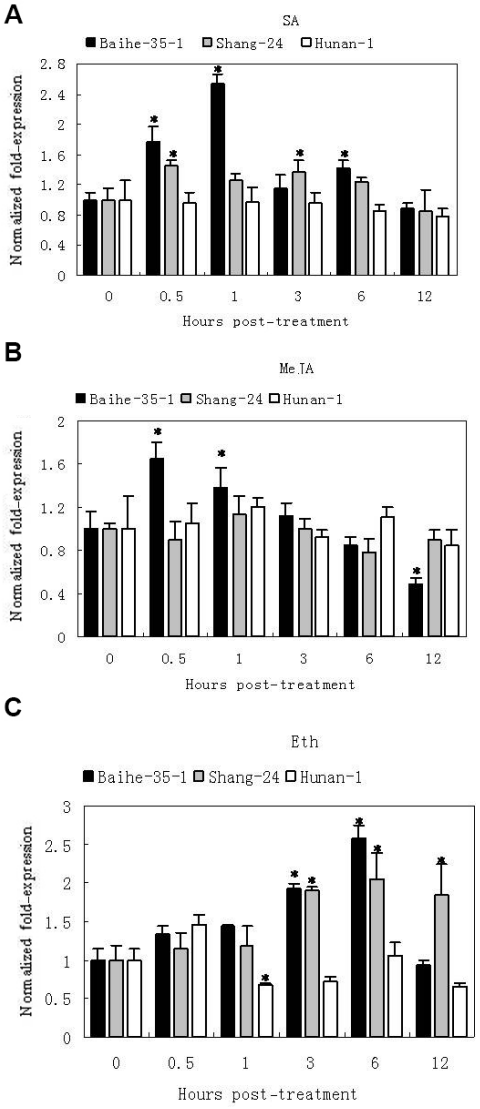

Figure 4. Expression profiles of *VTC* respond to exogenous plant defense signaling molecules. Expression of *VTC* response to salicylic acid (SA; **A**); methyl jasmonate (MeJA; **B**), and ethephon (Eth; **C**) treatment in the leaves of 'Baihe-35-1', 'Shang-24', and 'Hunan-1'. *Actin1* was used as internal control for qRT-PCR and fold-expressions indicate expression level in treated leaves of each genotype compared with the negative control, which was set to 1. Asterisks indicate a significant difference ($P < 0.05$) in *VTC* expression. Mean values and SDs were obtained from 3 technical and 3 biological replicates.

Expression profile of *VpVTC* in various organs and at different developmental stages of fruit

As revealed by semi-quantitative RT-PCR, the *VpVTC* gene was expressed not only in leaves, stems, inflorescence, and tendrils, but also in grapes at all developmental stages. The highest transcript level in the fruit was obtained at 35 daf, and afterwards, a dramatic decrease occurred until a minimum was reached at 65 daf (Figure 5).

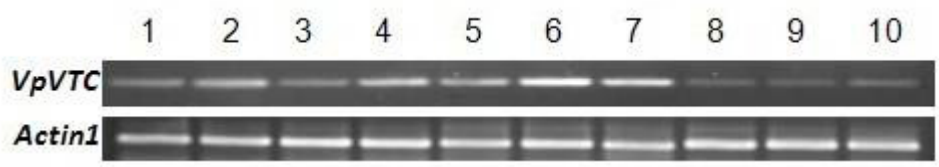

Figure 5. Expression pattern analysis of *VpVTC* in various organs and in fruit at different developmental stages by semi-quantitative RT-PCR. *Lane 1* = leaves; *lane 2* = stems; *lane 3* = inflorescence; *lane 4* = tendril; *lane 5* = fruit 20 days after flowering (daf); *lane 6* = fruit 35 daf; *lane 7* = fruit 50 daf; *lane 8* = fruit 65 daf; *lane 9* = fruit 80 daf; *lane 10* = fruit 95 daf.

Changes in AsA levels in leaves infected by *E. necator*

In order to determine the relationship between expression of *VTC* and the accumulation of vitamin C, we further compared the AsA concentration between the resistant genotype 'Baihe-35-1' and the susceptible genotype 'Hunan-1'. In 'Baihe-35-1', the AsA concentration increased gradually after inoculation and reached the maximum at 96 hpi and then decreased (Figure 6). In contrast, in the susceptible genotype 'Hunan-1', the AsA concentration did not vary significantly during the course of the experiment.

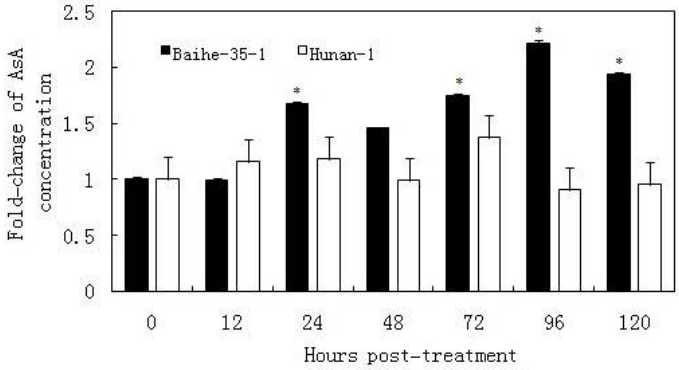

Figure 6. Changes of L-ascorbic acid (AsA) concentration in resistant genotype 'Baihe-35-1' and susceptible genotype 'Hunan-1' leaves induced by *Erysiphe necator*. Fold-changes were calculated relative to the AsA concentration in untreated leaves of 'Baihe-35-1' and 'Hunan-1'. Fold-expressions indicate expression level in treated leaves of each genotype compared with the negative control, which was set to 1. Asterisks indicate a significant difference ($P < 0.05$) in AsA concentration. Mean values and SDs were obtained from 3 technical and 3 biological replicates.

DISCUSSION

Sequence analysis of *VpVTC*

A. thaliana VTC2 encoding GGP is required for ascorbate biosynthesis. The protein is a member of the HIT superfamily (Dowdle et al., 2007; Müller-Moulé, 2008). The nucleotidylylated intermediate of HIT transferases is stable in water and awaits reaction with phosphate (phosphorolysis) or a specific phosphorylated substrate (transfer). Given the lack of conservation of the third His residue in the HIT motif of the plant *VTC2* sequence, and the requirement of an intact *VTC2* gene for maintenance of the ascorbate pool, it is evident that this motif contributes to catalyzed vitamin C biosynthesis in *Arabidopsis* (Linster et al., 2007). It is suggested that *Arabidopsis VTC2* and *VTC5* proteins, and their homologs in other plants, are enzymes that guanylylate a conserved active site His residue with GDP-L-galactose, forming L-galactose 1-phosphate for vitamin C synthesis, and regenerate the enzyme with phosphate to form GDP (Linster et al., 2008). *VpVTC* contains the same HIT motif as *Arabidopsis VTC2* (Figure 2). Multiple alignments of amino acid sequences indicated that *VpVTC* share high levels of similarity with reported *VTC* proteins (Figure 2). Therefore, *VpVTC* possibly encodes a novel GGP-like gene for vitamin C biosynthesis in the grapevine.

Expression profile of *VpVTC* in various organs

Fresh fruit are generally considered to be good sources of dietary AsA. However, several fruits such as grapes, apples, and pears, are also known to be low in ascorbic acid levels (Szeto et al., 2002). This variation may be related to expression of interrelated genes. A recent study demonstrated that expression of the *VTC2* gene was down regulated at least 16-fold as grape berries ripened from 14 daf to veraison (Melino et al., 2009). Similarly, the present study revealed that expression of *VpVTC* was lowest in the fruit from 50 daf to complete maturity (Figure 5).

Responses of *VpVTC* to PM pathogen and defense signaling molecules

Plants are attacked by many disease-causing organisms including bacteria, fungi, viruses, and nematodes. However, plants have developed a variety of sophisticated defense mechanisms to resist pathogen attacks. These complex defense mechanisms depend on expression of numerous proteins involved in the upregulation of antioxidants and in the activation of signaling pathways, for example. Here, we provide evidence suggesting for a role of *VpVTC* in protecting grapes against PM attack. We examined the expression pattern of *VTC* in 'Baihe-35-1', 'Shang-24', and 'Hunan-1' in response to *E. necator* infection. The results showed that expression of *VTC* was induced post-PM infection in all 3 grapevine genotypes tested and displayed higher and stronger expression levels in the resistant genotype 'Baihe-35-1' than in the susceptible genotype 'Hunan-1', specifically at 96 and 120 hpi, respectively (Figure 3). This result indicated that *VpVTC* was involved in active defense response. Although previous research on the direct relationship between *VTC* and disease resistance is sparse, other studies have considered its role in plant abiotic stress. The increase in leaf L-ascorbate content measured after a 24-h exposure to high light in *Arabidopsis* was

accompanied by increased expression of *VTC2* and *VTC5* and by a 20-fold increase in GGP activity (Dowdle et al., 2007). *Chlamydomonas reinhardtii* cells facing oxidative stress show increased abundance of *VTC2* transcripts as well as all of the enzymes of the ascorbate-glutathione system, and an increase in ascorbate content (Urzica et al., 2012).

Moreover, the existence of multiple defense strategies and complex signaling networks in plants leads to enhanced defense capacity (Rea et al., 2002). Induced defense responses are regulated by a network of inter-connecting signal transduction pathways, in which the hormonal signals SA, MeJA, and Eth play a major role. Application of SA, MeJA, and Eth can coordinately activate transcripts of different defense-related proteins (Lu et al., 2006). MeJA treatment increased expression of *VTC1* and *VTC2* transcripts and enhanced AsA accumulation in *Arabidopsis* (Sasaki-Sekimoto et al., 2005). SA application positively increased AsA content in pepper and strawberry leaves (Elwan and El-Hamahmy, 2009; Karlidag et al., 2009). The present study demonstrated that *VpVTC* was induced by SA, MeJA, and Eth in the resistant genotype 'Baihe-35-1', and the maximum induction occurred at 1, 0.5, and 6 h, respectively. These results indicated that the combined action of SA, MeJA, and Eth signal pathways involved in the regulation of defense responses in grape by upregulating the transcription of *VpVTC*.

The roles of AsA during pathogenesis

It is well known that SA, MeJA, and Eth are important secondary messengers of oxidative stress signaling. Indeed, AsA, as an antioxidant, is also involved in a complex phytohormone-mediated signaling network that links ozone and pathogen responses. Application of Eth increased AsA concentrations in apple peel and meiwa kumquat skin or flesh (Burden and Bramlage, 1994; Kondo et al., 2005). The higher levels of glutathione and AsA observed in SA-deficient plants may contribute to their alleviated symptoms (Wang et al., 2011a). In contrast, other studies have shown that these signaling molecules have no effect on AsA content (Keramat et al., 2009; Gergoff et al., 2010; Krajnc et al., 2011), suggesting that the effects of signaling molecules on AsA accumulation differ between species. The apple variety 'Prima' has a higher than expected L-AsA content at harvest, which is associated with improved resistance to necrotrophic pathogens. This suggests that the ability of fruit tissue to resist infection by necrotrophic pathogens may be specifically associated with aspects of cellular AsA metabolism, conceivably through the pool of apoplastic AsA, which forms part of the first line of defense against external oxidative injuries (Davey et al., 2007). Moreover, AsA content also increased in mycorrhizal strawberry after pathogen inoculation (Li et al., 2010b). Early and high-dose AsA treatment alleviates the symptoms of SA-deficient plants, and eventually inhibits RNA virus replication after 20 days (Wang et al., 2011a). In the present study, the AsA concentration of *E. necator*-inoculated leaves showed different tendencies in the resistant genotype 'Baihe-35-1' and the susceptible genotype 'Hunan-1' (Figure 6). The magnitude and direction of change in the AsA concentration matched changes in the transcript levels of *VTC*. Upregulating expression of *VpVTC* resulted in a gradual increase of the AsA concentration in leaves inoculated with *E. necator* of the resistant genotype 'Baihe-35-1' (Figure 6). AsA is one of the most abundant compounds in green leaves, and as an antioxidant, it plays an important role in maintaining cellular redox balance. These results suggest that elevated *VpVTC* transcripts may contribute to the synthesis of more AsA, which can depress the overproduc-

tion of ROS and maintain redox balance in PM-infected leaves. Previous research has shown that fruit was able to mount a rapid and efficient defense response and to maintain a generally higher L-AsA content after being infected by *B. cinerea* (Davey et al., 2007), which is in accordance with results of the present study. In contrast, the AsA-deficient *A. thaliana VTC1*, *VTC2*, *VTC3*, and *VTC4* mutants were more resistant to *P. syringae* relative to the wild-type (Pavet et al., 2005; Mukherjee et al., 2010).

However, Wang et.al. (2011a) confirmed that this enhanced resistance could be eliminated by 0.5 mM AsA treatment and that under a high-dose 5 mM AsA treatment, *VTC1* and wild-type plants display similar phenotypes. It was further suggested that symptom alleviation by high-dose AsA treatment and by AsA deficiency might be accomplished by different mechanisms (Wang et al., 2011a). This may result from the signaling role of pathogen-induced ROS in AsA-deficient *Arabidopsis* mutants, which results in the programmed cell death, i.e., hypersensitive response of plants against invading pathogens. Together, our results indicate that *VpVTC* is a very important gene in biosynthetic pathways for ascorbate and is likely to participate in the regulation of resistance to *E. necator* by inducing SA, MeJA, and Eth molecular signals.

It is still not well-understood whether induction of AsA by SA, MeJA, and Eth is a direct signaling response that requires all components of these molecules, or is triggered by an indirect effect of these molecules, such as ROS generation, which is caused by, but is not specific to, these signaling molecules. Further study using genetic approaches is needed in order to fully understand the role of AsA in regulating grapevine defenses against invading pathogens, and to determine the potential links between AsA and signaling molecules.

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