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Research paper

Ectopic expression of a *Ve* homolog *VvVe* gene from *Vitis vinifera* enhances defense response to *Verticillium dahliae* infection in tobacco

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ABSTRACT

Verticillium wilt is a soil borne disease that can cause devastating losses to the production of many economically important crops. A *Ve1* homologous gene responding to *Verticillium dahliae* infection was identified in *Vitis vinifera* cv. "HeiFeng" by semi-quantitative reverse transcription polymerase chain reaction and was designated as *VvVe*. The overexpression of *VvVe* in transgenic *Nicotiana benthamiana* plants significantly enhanced the resistance to isolate V991 of *V. dahliae* when compared with the wild type plants. The expressions of defense-related genes including the salicylic acid regulated gene pathogen-related 1 (*PR1*) but not *PR2*, the ethylene- and jasmonic acid-regulated genes ethylene response factor 1 (*ERF1*) and lipoxygenase (*LOX*) were significantly increased due to over expression of *VvVe*. And greater accumulation of active oxygen, callose and phenylalanine-ammonia lyase were observed in the leaves of transgenic *VvVe* tobacco plants than the wild type when under infection by *V. dahliae*. Moreover, the hypersensitive response mimicking cell death was exclusively occurred in the transgenic *VvVe* tobacco plants but not in the wild type. Taken together, the *VvVe* gene is a *Ve1* like gene which involves in the signal cascade of salicylic acid, jasmonate, and ethylene defense pathways and enhances defense response to *V. dahliae* infection in the transgenic tobacco.

1. Introduction

Verticillium wilt is a plant vascular disease caused by *Verticillium*, a genus of ascomycete fungi, which contains ten newly classified species including *Verticillium albo-atrum*, *Verticillium tricorpus*, *Verticillium zaregamsianum*, *Verticillium isaacii*, *Verticillium klebahnii*, *Verticillium nubilum*, *Verticillium dahliae*, *Verticillium longisporum*, *Verticillium alfalfae* and *Verticillium nonalfalfae* (Inderbitzin et al., 2011). Devastating losses of production occurred in many economically important crops such as cotton, tomato, pepper, and potato due to *Verticillium* wilt (Cai et al., 2009), because no effective controlling methods available for the disease and genetic variation in *Verticillium* caused by transposon and DNA (deoxyribonucleic acid) horizontal transfer (Amyotte et al., 2012; Jonge et al., 2012).

The first report of *Verticillium* wilt of grapevine (*Vitis vinifera*) caused by *V. dahliae* was made in 1977 in the United States, and later it was found in the Shihezi Region of Xinjiang Province in China in 2007 (Schnathorst and Goheen, 1977; Zhang et al., 2009). The *Verticillium* wilt of *V. vinifera* arouses more attention as it presents a serious and uncontrollable threat. Therefore, cloning of resistance genes and elucidating of the resistance mechanism are both significant and urgent.

The *Ve* gene of tomato (*Solanum lycopersicum*) was the first cloned *Verticillium* wilt resistance gene, which involves two inversely oriented genes, *Ve1* and *Ve2*, within its locus, but only *Ve1* owns the resistance function (Fradin et al., 2009). The *Ve1* gene belongs to the receptor-like proteins (RLPs) located on the membrane and has the extracellular Leu-rich repeat (eLRR). RLPs recognize and bind the pathogen proteins through LRRs and participate in the signal transduction of disease resistance. Some RLPs have been well characterized, such as the *Cf* resistance genes of tomato (Jones et al., 1994; Thomas et al., 1997; Dixon et al., 1998), *LeEIX* genes in tomato (Ron and Avni, 2004), and *Vf* genes in apple (Durel et al., 2003; Guérin et al., 2007). Several *Ve* homologous genes that confer *Verticillium* wilt resistance have been reported, such as *GbVe* (Zhang et al., 2011) and *GbVe1* (Zhang et al., 2012) from island cotton, *SlVe1* in *Solanum lycopersicoides* (Chai et al., 2003), *StVe* from *Solanum torvum* Swartz (Fei et al., 2004), and *mVe1* from *Mentha longifolia* (L.) Huds (Vining and Davis, 2009). Very few genes with resistance to *Verticillium* wilt of grapevine have been reported. Vv-AMP1, a 77-amino-acid peptide and berry specific of *V. vinifera*, showed antifungal activity against a broad spectrum of plant pathogenic fungi, such as *Fusarium oxysporum* and *V. dahliae* (De Beer and Vivier, 2008).

Abbreviations: ET, ethylene; *ERF1*, ethylene response factor 1; eLRR, extracellular Leu-rich repeat; HR, hypersensitive response; JA, jasmonate; *LOX*, lipoxygenase; PTI, pathogen-associated molecular pattern-triggered immunity; *PR*, pathogenesis-related; PAL, phenylalanine-ammonia lyase; RLPs, receptor-like proteins; RT-PCR, reverse transcription-polymerase chain reaction; SA, salicylic acid; WT, wild type.

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In this study, a *Ve* homologous gene in grapevine, *VvVe* (XP_002263233.1), was strongly induced by *V. dahliae* infection. Over expression of *VvVe* in *Nicotiana benthamiana* induced the expression of pathogenesis-related 1 (*PR1*), ethylene response factor 1 (*ERF1*) and lipoxygenase (*LOX*) as well as greater accumulation of active oxygen, callose, phenylalanine-ammonia lyase (PAL) and hypersensitive response (HR) mimicking cell death, thus making the plants resistant to *Verticillium* wilt.

2. Materials and methods

2.1. Plants, V. dahliae strain and inoculation method

Plantlets of *V. vinifera* cv. "HeiFeng", "Xiahei" and "Alexander" were obtained from the grape germplasm repository of Jiangsu Province in China. *N. benthamiana* wild type (WT) and transformed plants were grown in vermiculites under 25 °C during the day and 21 °C at night with 60–70% relative humidity on a 16-h light/8-h dark cycle. The strong virulence defoliating isolate V991 of *V. dahliae* (Zhang et al., 2012) was activated on potato dextrose agar at 25 °C and cultured in Czapek liquid in a swing bed. Before inoculation, the medium was filtered with gauze and the spores of *V. dahliae* were counted with a hemocytometer. For *V. dahliae* inoculation, the grape plantlets were inoculated by root irrigating with 1×10^7 spores per pot (Zhang et al., 2012), while plants of *N. benthamiana* with two true leaves were uprooted, dipped for 5 min in a suspension containing 1×10^7 spores/ml, and replanted in the vermiculites.

2.2. Isolation and expression analysis of Ve homologous genes in grapevine

Ve homologous proteins in grapevine were identified by blastp search grapevine proteins in NCBI with tomato Ve1 (GenBank: AAK58682.1) as a query. Phylogenetic tree was constructed by MEGA6 (Tamura et al., 2013). The putative motif and domains were identified by SMART (http://smart.embl-heidelberg.de/). The expression levels of four random selected *Ve* homologous genes (XP_002263233.1, CAN78183.1, CBI21723.3 and XP_002267585.2) in response to *V. dahliae* inoculation were further tested at 4 days post-inoculation by semi-quantitative reverse transcription polymerase chain reaction (semi-quantitative RT-PCR) and real-time RT-PCR. The gene-specific primers for semi-quantitative RT-PCR and real-time RT-PCR and their accession numbers are listed in Table 1. The grape actin gene (XM_002282480.3) was used as an internal control. Total RNA was extracted from leaf tissues with an RNAiso Kit (TaKaRa, Daliang, China) and transcript into cDNA using a Primscript RT-PCR kit (TaKaRa) according to the manufacturer's instructions. The semi-quantitative RT-PCR was performed as follows: 94 °C for 4 min, 24–30 cycles of 94 °C for 30 s, 55 °C for 40 s and 72 °C for 2 min. The real-time RT-PCR reaction was conducted with SYBR Green I (TaKaRa) at real-time PCR thermal cycler qTOWER 2.0/2.2 (Analytik Jena, Germany) with a program contained an initial denaturation step of 1 min at 95 °C, followed by denaturation for 15 s at 95 °C, annealing for 20 s at 60 °C, and extension for 20 s at 72 °C for 40 cycles. The relative expression levels of the selected transcripts were calculated using the $2^{-\Delta\Delta Ct}$ method with three biological replicates.

2.3. Resistance assessment of transgenic VvVe tobacco to V. dahliae

The full ORF of *VvVe* was amplified from *V. vinifera* cv. "HeiFeng" with primers VvVe-F/VvVe-R and constructed into a plant expression vector pK2GW7.0. Transgenic *VvVe* tobacco plants were obtained by *Agrobacterium*-mediated transformation of leaf disc of *N. benthamiana* (Horsch et al., 1985). Four independent transgenic lines were random chosen to assess for their *V. dahliae* resistance using the root dip method as described above. Disease grades were defined as following: level 0, no disease plant; level 1, 0.1–25% incidence in the plant leaves; level 2, 25–50% incidence in the plant leaves; level 3: 50–75% incidence in the plant leaves; and level 4, >75% incidence in the plant leaves. The disease index = [(∑ - disease grades × number of infected) / (total checked plants × 4)] × 100. At least 40 plants of each transgenic line were tested. Observation of the disease index started at 15 days after the *V. dahliae* inoculation.

Table 1
Primers used in this study.

Name of primers	Targeted gene (GenBank number)	Sequence (5′–3′)
NbEF1a-QF	EF1a (AY206004.1)	AGAGGCCCTCAGACAAAC
NbEF1a-QR		TAGGTCCAAAGGTCACAA
Prb-1-QF	PR1 (XM_009607401.1)	GTGGACACTATACTCAGGTG
Prb-1-QR		TCCAACTTGGAATCAAAGGG
Pr2b-QF	PR2 (XM_009762002.1)	AGGTGTTTGCTATGGAATGC
Pr2b-QR		TCTGTACCCACCATCTTGC
ERF1-QF	ERF1 (XM_009613506.1)	GCTCTTAACGTCGGATGGTC
ERF1-QR		AGCCAAACCCTAGCTCCATT
LOX-QF	LOX (XM_009612605.1)	AAAACCTATGCCTCAAGAAC
LOX-QR		ACTGCTGCATAGGCTTTGG
Grape-1-F	XP_002263233.1,	ATGGTCACATAAGCGGTTCAAAT
Grape-1-R	semi-quantitative RT-PCR	TCAGACTTGTCAACTCCCTTGGA
Grape-2-F	CAN78183.1,	CTCCCATGCAGTTGACAGGA
Grape-2-R	semi-quantitative RT-PCR	TGGCCTCTATGGCACCATTC
Grape-3-F	CBI21723.3, semi-quantitative	ATGGTGCATTGCATGGGAGA
Grape-3-R	RT-PCR	CAACCGGGCATAGCCAAAAC
Grape-4-F	XP_002267585.2,	ACAAAGGCTCAACCTGGCTT
Grape-4-R	semi-quantitative RT-PCR	TCTCCCATGCAATGCACCAT
Grape-actin-F	actin (XM_002282480.3),	TTGCCATTCAGGCTGTTCTTTCT
Grape-actin-R	semi-quantitative RT-PCR, real time RT-PCR	ATAGTAATGACTTGCCCATCAGG
VvVe-F	XP_002263233.1, cloning	ATTATAAGGGAACCTTTGGGTATA
VvVe-R	gene	AGTATCATTCACCACAAAATACGA
VvVe-QF	XP_002263233.1, real time	CTTTCACGCAATGATTTCTCTGGT
VvVe-QR	RT-PCR	TGATGATGGAATATGCCCTGTAAGA

2.4. Expression analysis of defense- related genes in transgenic VvVe tobacco

At 4 days post-inoculation by *V. dahliae*, the expression levels of four internal defense-related genes *PR1*, *PR2*, *ERF1* and *LOX* in tobacco were analyzed by real-time RT-PCR as described above, using the *NbEF1a* gene (AY206004.1) as the internal standard. Specific primers for these genes were list in Table 1.

2.5. Detection of reactive oxygen, callose, PAL and cell death formation in transgenic VvVe tobacco

Tobacco leaves were inoculated with the 10 μl spore drop of V991 (1×10^7 spores/ml) and then incubated at 25 °C in a moist incubator for 12 h–6 days. Histochemical assay of reactive oxygen accumulation, callose deposition and cell death in tobacco leaves was performed according to the method of Choi et al. (2012). While the PAL activity was measured by the method of Gayoso et al. (2010). A unit of PAL activity was defined as the amount of enzyme that increases 0.01 of absorbance at 290 nm per hour under the assay conditions.

3. Results

3.1. VvVe gene identification in grapevine

By blastp search grapevine proteins in NCBI, 100 hit sequences producing significant alignments with tomato Ve1 were identified in grapevine and constructed into the phylogenetic tree by MEGA6 (Fig. 1a). The gene expression level in response to *V. dahliae* inoculation was tested in four genes from two independent phylogenetic clusters in three grapevine genotypes. Semi-quantitative RT-PCR revealed that XP_002263233.1 was up-regulated in cv. "Heifeng" and "Alexander" but not in cv. "Xiahei" after *V. dahliae* inoculation (Fig. 1b). By contrast, CBI21723.3, CAN78183.1 and XP_002267585.2 were down-regulated