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Grape apoplasmic β-1,3-glucanase confers fungal disease resistance in *Arabidopsis*

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ABSTRACT

To identify apoplasmic proteins in grape cells, we investigated proteins secreted into grape cell culture medium and detected β-1,3-glucanase VvGHF17 in the medium. *VvGHF17* is expressed constitutively in grape leaves and berry pulp and skin. Experiments using VvGHF17:GFP fusion protein demonstrated that VvGHF17 is localized at the apoplasmic space of cells, suggesting that VvGHF17 is apoplasmic β-1,3-glucanase. To understand how VvGHF17 protects grapevine against phytopathogens, we created *Arabidopsis* transgenic plants expressing VvGHF17. VvGHF17-expressing *Arabidopsis* plants exhibited disease resistance to both *Botrytis cinerea* and *Colletotrichum higginsianum*, but not to *Pseudomonas syringae* pv. tomato DC3000. The inhibitory effect of VvGHF17 to the infection by the fungi was promoted when VvGHF17 protein was expressed in large amounts in the plants. In conclusion, apoplasmic VvGHF17 may be a candidate for the transgene to create transgenic grapevines that are robust to multiple fungal attacks.

1. Introduction

Plants have acquired a number of resistance machineries that enable them to withstand infection by a variety of phytopathogens. The production of pathogenesis-related proteins (PRs) is an example of a well-studied resistance machinery (van Loon et al., 2006). PRs are induced in many plant species in response to attack by plant pathogens, including fungi, bacteria, viruses, and insects. At present, there are 17 PR families (PR-1 to PR-17) that are classified in terms of protein sequence, immunological property, and enzymatic activity, irrespective of plant species (Fritig et al., 1998).

Some PRs exhibit antimicrobial activities that stem from their ability to hydrolyze fungal cell wall components (Adams, 2004). β-1,3-Glucanase (glucan endo-1,3-β-D-glucosidase, EC 3.2.1.39) is categorized under the PR-2 family. It digests 1,3-β-D-glucosidic linkages in β-1,3-glucans, which are the components of the cell walls of fungal hyphae and germ tubes. The PR-2 family members are grouped into four classes, I–IV, according to molecular size, isoelectric point, and subcellular localization. Class I β-1,3-glucanases are localized in vacuoles, whereas classes II–IV β-1,3-glucanases are secreted into the apoplasmic spaces of plant cells (Ebrahim and Singh, 2011).

The role of plant β-1,3-glucanase in increasing plant resistance against phytopathogens has been described for several plant species (Chen et al., 2006; Singh et al., 2014). β-1,3-Glucanase purified from grape leaves inhibited the growth of germ tubes of grape powdery mildew *in vitro* (Giannakis et al., 1998), demonstrating that β-1,3-glucanase has antifungal activity and that the production of β-1,3-glucanase is one of the grapevine's defense mechanisms against powdery mildew. Methyl jasmonate, an exogenous elicitor, upregulated β-1,3-glucanase gene transcription in grape leaves, thereby resulting in the protection against grape powdery mildew in vineyards (Belhadj et al., 2006). In contrast, β-1,3-glucanase, which is naturally present in berry skins, may be not sufficient to protect grape berries against fungal pathogens. β-1,3-Glucanase expression was increased in berry skins during berry ripening and was still abundant at harvest (Deytieux et al., 2007). However, the ripe berries showed increased susceptibility to bunch rot disease (Commenil et al., 1997). Thus, in grapevine, the role of β-1,3-glucanase on increasing plant resistance against phytopathogens is uncertain.

Abbreviations: nano-LC–MS/MS, nanoscale liquid chromatography coupled to tandem mass spectrometry; PR, pathogenesis-related protein.

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A molecular evaluation of grape β-1,3-glucanase has to be conducted to understand how it protects grapevine against phytopathogens. One of the strategies to this end is the creation of β-1,3-glucanase-overexpressing plants. The objectives of the study are: (1) to characterize apoplasmic β-1,3-glucanase in grapevine, and (2) to assess its inhibitory effect on the infection caused by phytopathogens in β-1,3-glucanase-overexpressing *Arabidopsis* plants.

2. Materials and methods

2.1. Plant materials

Grape cell cultures prepared from the meristems of *Vitis vinifera* cv. Koshu were used (Katoh et al., 2009) and maintained in modified Gamborg's B5 medium at 27 °C.

Grapevines of *V. vinifera* cv. Koshu were cultivated in the experimental vineyard of The Institute of Enology and Viticulture, University of Yamanashi, Yamanashi, Japan (latitude, 35°40′N; longitude, 138°33′E; elevation, 273 m). Grapevines were trained to bilateral cordons with eight to ten shoots on each cordon. To detect the gene expression of apoplasmic β-1,3-glucanase in the field, the following organs were collected from the grapevines. The tenth leaves from the bottom of shoot having 12–16 leaves were used as leaf samples. Berry samples were collected two weeks after flowering (June 11, 2014). Grape berries were dissected and skins, pulps, and seeds were collected as individual organ samples.

2.2. Identification of proteins secreted by grape cell culture

Grape cell culture was carried out in modified Gamborg's B5 medium at 27 °C for one week. The supernatant from the culture medium was collected by centrifugation at 10,000 × *g*, 4 °C for 10 min, and subjected to 12.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the gel was stained with Coomassie brilliant blue (CBB) solution (Bio-Rad, Hercules, CA). Three major single bands on the gel were excised, and then subjected to nanoscale liquid chromatography coupled to tandem mass spectrometry (nano-LC–MS/MS) by Japan Bio Services Co., Ltd. (Saitama, Japan). Sequence analysis of the digested peptides was performed using MASCOT search engine (http://www.matrixscience.com) based on MS/MS ion search (Perkins et al., 1999).

2.3. Total RNA isolation

Leaves, skins, pulps, and seeds of grapevine were used for total RNA isolation. Total RNA was isolated as described previously (Kohno et al., 2012).

Rosette leaves of 34-day-old *Arabidopsis* plants were homogenized with an SK mill (SK-200, Tokken, Kashiwa, Japan) after freezing with liquid nitrogen. Total RNA isolation from the pulverized samples was performed using RNAiso Plus (Takara, Otsu, Japan) according to the manufacturer's instructions.

2.4. Real-time RT-PCR analysis

First-strand cDNA was synthesized from total RNA using a PrimeScript RT Reagent Kit with gDNA Eraser (Takara), and then real-time PCR was performed using an SYBR Premix Ex Taq II (Takara). Real-time RT-PCR conditions were as follows: 37 °C for 15 min for RT reaction and 85 °C for 5 s for cDNA synthesis, and then 40 cycles at 95 °C for 5 s and at 60 °C for 30 s for PCR amplification. Nucleotide sequences of the primers used in this study were as follows: VvGHF17 primers (5′-TTCATGGGTCCAAGACAACA-3′ and 5′-AGCCACTTGATGGTGGGTAG-3′, corresponding to bases 345–364 and 568–549 of *V. vinifera* β-1,3-glucanase (LOC100233076), GenBank accession no. XM_002277410, respectively), grape β-actin primers (5′-CAAGAGCTGGAAACTGCAAAGA-3′ and 5′-AATGAGAGATGGCTGGAAGAGG-3′, corresponding to bases 409–430 and 537–516 of *V. vinifera* β-actin, GenBank accession no. AF369524, respectively), and *Arabidopsis* actin primers (5′-GCCGACAGAATGAGCAAAGAG-3′ and 5′-AGGTACTGAGGGAGGCCAAGA-3′, corresponding to bases 1098–1118 and 1224–1204 of *Arabidopsis thaliana* actin 1 mRNA, GenBank accession no. NM_179953, respectively). The dissociation curves for each sample were analyzed to verify the specificity of the amplification reaction. Each actin was used for normalization and *VvGHF17* expression levels of each sample were expressed as a relative value.

2.5. Subcellular localization of VvGHF17 in onion epidermal cells

The open reading frames of *VvGHF17* were amplified from leaf total RNA by RT-PCR with the following primers: 5′-GGTACCATGGCTAAGCTAAGCTCTATTCAGC-3′ containing a *Kpn*I site (underlined) and 5′-CTCGAGGTTGAAATTGATTGTGTATT-3′ containing an *Xho*I site (underlined). The PCR product was digested with *Kpn*I and *Xho*I and ligated into the *Kpn*I and *Xho*I sites of the GFP expression plasmid that was previously constructed (Takato et al., 2013), resulting in the VvGHF17:GFP expression plasmids. Onion epidermal cell monolayers were bombarded with the expression plasmids using a particle gun (PDS-1000/He, Bio-Rad). After incubation at 27 °C for 24 h in the dark, GFP fluorescence in the onion epidermal cells was observed under a fluorescence microscope (Olympus, Melville, NY). The images were processed using cellSens Dimension Software ver. 1.4 (Olympus).

2.6. Expression of VvGHF17 in Arabidopsis plants

The open reading frames of *VvGHF17* were amplified from leaf total RNA by RT-PCR. The nucleotide sequences of the primers used were as follows: 5′-CATATGGCTAAGCTCTATTCAGC-3′ containing an *Nde*I site (underlined) and 5′-GGATCCTTTGTCGTCGTGGTCTTTGTAGTCGTTGAAATTGATTGTGTATT-3′ containing a *Bam*HI site (underlined). The PCR products were digested with *Nde*I and *Bam*HI, followed by ligation to the *Nde*I and *Bam*HI sites of the binary vector pRI101-AN (Takara). The transformation vector was introduced into *Agrobacterium tumefaciens* strain LBA4404. *A. thaliana* Col-0 was transformed with *Agrobacterium* by the floral dip method (Clough and Bent, 1998). The transformants were screened on 1 × Murashige and Skoog (MS) medium plates containing 50 μg kanamycin/l. Three independent T3 homozygous transformants, denoted by GHF17-OE1, GHF17-OE2, and GHF17-OE3, were obtained. The expression levels of *VvGHF17* in each transformant were measured by real-time RT-PCR. As the control transgenic plant, a homozygous line transformed with pRI101-AN vector was used and denoted by pRI.

2.7. Resistance to disease caused by fungal infection

T3 homozygous seeds were plated on MS medium and incubated at 22 °C for 10 days in an incubator (11.8 Wm^{-2}/16 h/day). Then, the seedlings were planted in soil. *Botrytis cinerea* wild isolate, isolated from the infected grape berry, was maintained on potato dextrose agar (PDA) for 14 d. Spores on the PDA were collected by washing with sterilized water and a spore suspension was prepared at 4.5×10^5 spores/ml as inoculum. Three rosette leaves of each potted plant (three replications) at day 34 after sowing were punctured by a needle to make a wound. One hundred microliters of the spore suspension was dropped on the wound. The plants were incubated at 22 °C for 29 days in a moisture chamber and then incubated in