Characterization and Expression of β -1,3-Glucanase Genes in Jujube Fruit Induced by the Microbial Biocontrol Agent *Cryptococcus laurentii*

Shi P. Tian, Hong J. Yao, Xin Deng, Xiang B. Xu, Guo Z. Qin, and Zhu L. Chan

All authors: Key Laboratory of Photosynthesis and Molecular Environment Physiology, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China; and second, fourth, and sixth authors: Graduate School of the Chinese Academy of Sciences, Beijing 100049, China.

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ABSTRACT

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Two β -1,3-glucanase genes were cloned from jujube (*Ziziphus jujuba* Mill) fruit and designated *Glu-1* and *Glu-2* (GenBank accession numbers DQ012940 and DQ093571), respectively. The expression of *Glu-1* and *Glu-2* in jujube fruit in response to wounding and microbial biocontrol agent was evaluated by semi-quantitative reverse-transcription polymerase chain reaction analysis. Wounding and treatment with *Cryptococcus laurentii* stimulated an increase in β -1,3-glucanase (EC 3.2.1.39) activity in jujube fruit. Analysis of gene expression proved that *Glu-1* was

highly induced both by wounding and *C. laurentii*, whereas *Glu-2* was broadly not responsive to the yeast. The expression of *Glu-1* was noticeably enhanced with increased concentrations of *C. laurentii*, suggesting that *Glu-1* may play a role in defense responses to fungal pathogens. The results hold true at the levels of gene activation and enzyme accumulation in jujube fruit treated by both stimuli, resulting in a significant decrease in disease incidence and lesion diameter, thus providing evidence that changes in β -1,3-glucanase activity are related to expression of the genes. Taken together, these findings suggest the possible use of β -1,3-glucanase activity as a biochemical marker for screening jujube fruit against fungal pathogens, and also provide a mechanistic framework for the functions of β -1,3-glucanase in defense responses.

Plants have developed a range of defense mechanisms against pathogen attack, including the induction and accumulation of pathogenesis-related (PR) proteins, such as β -1,3-glucanases and chitinases (11). PR proteins are strongly induced in response to wounding or infection by pathogens and accumulate abundantly at the site of infection, contributing to systemic acquired resistance (29,40). The defense response in plants is complex and involves both structural and biochemical barriers (42). β -1,3-Glucanase hydrolyses β -1,3-linked glucans, a structural component of fungal cell walls, and therefore is considered to be involved in the plant defense mechanism against fungal infection (7). β -1,3-Glucanases have been reported from many different plant species in relation to development and in response to a variety of stimuli, including growth regulators and chemical elicitors (24,34). Also, Chang et al. (5) reported that expression of β -1,3-glucanases was induced by fungal infection.

Previous studies have demonstrated that β -1,3-glucanases are associated with plant reproductive biology and PR defense (2,29,38,56). For example, β -1,3-glucanase genes (*TomPR-Q'a* and *TomPR-Q'b*) have been cloned from viroid-infected tomato plants (9). Characterization and expression of β -1,3-glucanase genes in defense response to pathogens also has been carried out in peach fruit (25,44). β -1,4-Glucanases are highly expressed in strawberry (31) and peach (46), supporting a role in fruit softening.

Recently, microbial biocontrol agents have been shown to elicit defense mechanisms of the host (10,12). Production of cell-wall

lytic enzymes and induction of host resistance are considered to be an important action of microbial biocontrol agents against pathogenic fungi (17,32). Biocontrol efficiency of antagonists may be related to their ability to induce PR proteins in the host (22). Previous experiments have indicated that *Pichia membranaefaciens* and *Candida guilliermondii* are able to induce chitinase and β -1,3-glucanase activity in vitro, and to induce an increase in β -1,3-glucanase and chitinase activities in the wounds of nectarine fruit, resulting in beneficial control for the disease caused by *Rhizopus stolonifer* (14). However, it is not fully understood how biocontrol agents induce β -1,3-glucanase and block fungal attack. Moreover, little information exists as to whether β -1,3-glucanase activity present in fruit tissues actually exhibits sufficient anti-fungal activity or accumulates to a high enough level to block fungal attack.

In previous studies, *Penicillium expansum* and *Alternaria alternata* were reported to be major pathogens causing post-harvest decay of jujube fruit in storage (39,45). The objectives of this work was, first, to investigate the inhibitory effects of *Cryptococcus laurentii* on *P. expansum* and *A. alternata* both in vitro and in jujube fruit; second, to measure population dynamics of the yeast with and without the fungal pathogens in the wounds of jujube fruit at different temperatures; and, last, to determine the effects of *C. laurentii* inoculation on β -1,3-glucanase activity in jujube fruit. Two β -1,3-glucanase genes were cloned from jujube fruit using reverse-transcription polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE) techniques. Characterization and expression of the genes in jujube fruit after wounding and the yeast inoculation with different concentrations was evaluated by semi-quantitative RT-PCR analysis. The possible mechanisms of β -1,3-glucanase involved in fruit defense responses to fungal pathogens then are discussed.

Corresponding author: S. P. Tian; E-mail address: tsp@ibcas.ac.cn

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MATERIALS AND METHODS

Fruit and fungi. Jujube fruit (*Ziziphus jujuba* Mill. cv. Dongzao) were harvested at commercial maturity from an orchard in Binzhou, Shandong Province, China, and transported to Beijing the day after harvest. The fruit were sorted based on size without physical injuries or infections, disinfested with 2% (vol/vol) sodium hypochlorite for 2 min, washed with tap water, and air dried prior to use.

Fungal pathogens *P. expansum* and *A. alternata* were isolated from decaying jujube fruit and cultured on potato dextrose agar at 25°C. Spores (2 weeks old) of the fungi were obtained by flooding the cultures with sterile distilled water containing 0.05% (vol/vol) Tween 80. The suspensions were filtered through three layers of sterilized cheesecloth and adjusted to a concentration of 1×10^5 spores/ml with the aid of a hemacytometer.

C. laurentii was isolated from the surface of peach fruit following the method described by Wilson et al. (51) and identified by CABI Bioscience Identification Services (International Mycological Institute, UK). The yeast was cultured in potato-dextrose broth (PDB) and adjusted to different concentrations of 1×10^7 and 1×10^8 CFU/ml according to the previous experiment (13).

Biocontrol assay with different concentrations of yeast. The fruit were wounded at the equator using a sterile nail. Aliquots of 20 μ l of a suspension of *C. laurentii* at 1×10^7 or 1×10^8 CFU/ml or sterile distilled water (as the control) were pipetted into each wound site (uniformly 4 mm deep by 3 mm wide). After 4 h, 20 μ l of a spore suspension of *P. expansum* or *A. alternata*, containing 1×10^5 spores/ml, was inoculated into the wound. All fruit were placed in plastic boxes (250 by 150 by 50 mm), then put in plastic film bag to maintain high relative humidity ($\approx 95\%$), and stored at 25 and 0°C. Disease incidence and lesion diameter caused by *P. expansum* and *A. alternata* were determined after 4 days at 25°C and 25 days at 0°C. Disease incidence was scored according to the number of decayed fruit. Each treatment was replicated three times with 10 fruit per replication and the experiment was conducted twice.

Population dynamics of *C. laurentii*. One wound was made on each fruit. Then, 15 μ l of a cell suspension of *C. laurentii* at 1×10^7 CFU/ml alone or in combination with 15 μ l of a spore suspension of *P. expansum* or *A. alternata* at 1×10^5 spores/ml were put into each wound. Inoculated fruit were placed in plastic film bags or stored at 25 and 0°C. Wound tissue was removed at different times according to the experiment following the method of Usall et al. (47) with some modifications. The resulting cylinders of excised tissue (10 mm deep by 10 mm in diameter) from four fruit were placed in a mortar with 10 ml of 0.05 M sodium phosphate buffer at pH 7.0 and ground with a pestle. Serial 10-fold dilutions were made in phosphate buffer. Then, 0.1 ml of each dilution was spread on nutrient yeast dextrose agar medium in petri plates and inoculated at 25°C. The colonies were counted at 48 h and expressed as $\log_{10}$ CFU per wound. Each treatment contained three replicates with 20 fruit per replicate, and the experiment was repeated twice.

Evaluation of spore germination of fungal pathogens. The suspensions of *P. expansum* and *A. alternata* were prepared as described above. Aliquots of 100 μ l of the pathogen suspensions at $\approx 1 \times 10^5$ spores/ml and 100 μ l of cell suspension of *C. laurentii* at 1×10^7 CFU/ml were transferred to a glass tube (180 by 25 mm) containing 5 ml of PDB. All tubes were placed on a rotary shaker at 100 rpm at 25°C. After 12 h of incubation, ≈ 200 spores of each pathogen were measured for germination rate and germ tube elongation. Spores were considered germinated when germ tube length was equal to or greater than spore length. Each treatment was replicated three times and the experiment was repeated twice.

Determination of β -1,3-glucanase activity. Jujube fruit were treated by two methods to determine β -1,3-glucanase activity. In

the wounding and inoculation experiment, there were three treatments, including (i) wounding alone, (ii) wounding and inoculation with 20 μ l of suspension of *C. laurentii* at 1×10^8 CFU/ml, and (iii) nonwounding and noninoculation (as the control). In the second experiment, jujube fruit were dipped in the suspensions of *C. laurentii* at different concentrations of 1×10^6 , 1×10^7 , and 1×10^8 CFU/ml, or in sterile distilled water (as the control) for 5 min. The second method of inoculation (by dipping in the suspension of *C. laurentii*) is like commercial treatment, because jujube fruit are easily injured in harvest and, in general, there are some small wounds on the surface of the fruit. All fruit were stored in the same conditions as described above. Each treatment was applied to three replicates of 20 fruit each. Treatments were arranged in a randomized complete block design, and the experiment was repeated twice.

All extract procedures were conducted at 4°C. β -1,3-Glucanase was assayed by measuring the amount of reducing sugar released from the substrate by the dinitrosalicylate method (21) with some modifications. Flesh samples (10 g) from 10 fruit were ground with 0.3 g of polyvinyl pyrrolidone in 30 ml of sodium acetate buffer (50 mmol per liter, pH 5.0) at 4°C. Then, 250 μ l of enzyme preparation was incubated with 250 μ l of 0.5% laminarin (wt/vol) for 1 h at 37°C. Next, 200 μ l of sterile distilled water was added to 50 μ l of the reaction mixture. The blank was the crude enzyme preparation mixed with laminarin with zero time incubation. The reaction was stopped by adding 250 μ l of 3,5-dinitrosalicylate and boiling for 5 min in a water bath. The solution was diluted with 4 ml of distilled water and the amount of reducing sugars was measured spectrophotometrically at 500 nm using a UV-160 spectrophotometer (Shimadzu, Japan). Specific activity of β -1,3-glucanase was expressed as the formation of glucose equivalents at 1 μ mol/h/mg of protein.

Gene cloning by RT-PCR and RACE. Total RNA was isolated from jujube fruit 24 h after wounding according to Kay et al. (23). Reverse transcription (RT) was carried out with oligo (dT)₁₈ primer and AMV reverse transcriptase (TaKaRa, Dalian, China). The conserved domain of β -1,3-glucanase gene was amplified by PCR performed with the synthesized cDNA, *Taq* DNA polymerase, and a pair of degenerate primers as follows: 5'-(A/G)TTGC(G/T)GT(A/T/G)GGAAATGAAG-3' (forward primer) and 5'-CAAG(A/T)GC(C/G)G(A/C)GTAAACAGC-3' (reverse primer). PCR cycling parameters were as follows: 94°C for 2 min; 6 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min; 25 cycles of 94°C for 30 s, 60 to 50°C for 30 s, and 72°C for 1 min; 8 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min; and, finally, 72°C for 10 min. PCR product was cloned into pGEM-T easy vector (Tiangen, Beijing) according to the manufacturer's instruction, and sequenced.

Full-length coding sequences for β -1,3-glucanase genes were obtained by 3' and 5' RACE. For 3' RACE, RT was carried out as described above using an adaptor oligo (dT) 3' primer (5'-GGCTCGAGTTTTTTTTT-3'). PCR then was performed using a gene-specific 5' primer (5'-GCTTACCCCTTTTCGCTTACA-3') designed according to sequence of the cloned conserved domain and the adaptor primer. Two PCR products were cloned and sequenced as described and were named *Glu-1* and *Glu-2*. For 5' RACE of *Glu-1*, RT was performed with a 3' gene-specific primer (GSP1: 5'-TATGCCTTAACAACATTC-3') designed according to the sequence obtained from the 3' RACE fragment of *Glu-1*. PCR was carried out with the second gene-specific primer (GSP2: 5'-GAAGCCCATCCAGTCTCTGTAACAAC-3') and the anchor primer-1 (5'-GGCCACGCGTCTGACTAGTACG₍₁₈₎-3'). In order to improve specificity, a further round of PCR was performed with a nested gene-specific primer (GSP3: 5'-TTAATATCAATATTCTCCGATCACTC-3') and an anchor primer-2 (5'-GGCCACGCGTCTGACTAGTAC-3'). The resulting PCR product was cloned and sequenced. To obtain the full-length sequence of the 5' region of *Glu-1*, a second round of 5' RACE was performed as described

above with another set of 5' gene-specific primers (GSP4: 5'-GGGAGTCCAACCACCAAT-3', GSP5: 5'-ATTCAAGACCAGTTCCACTAAAGG-3', and GSP6: 5'-CTTCATCAGGTGAGGGTATGTTATC-3').

The 5' coding sequence for *Glu-2* was obtained by 5' RACE as described above with three *Glu-2* gene-specific primers designed according to the sequences of obtained 3' fragment of *Glu-2* and the conserved domain: 5'-TGGAACAGAGCGTAGGC-3', 5'-AATGGGAGGAGTTGTCG-3', and 5'-ATTTTGATGGAGTCGTTGAGGTTG-3'.

Semi-quantitative RT-PCR. Total RNAs were obtained at 0, 12, 24, and 48 h from jujube fruit treated with *C. laurentii* at concentrations of 10^8 CFU/ml or from jujube fruit treated with *C. laurentii* at 10^6 to 10^8 CFU/ml for 48 h. RT reactions were carried out using 2 µg of DNase-treated total RNA, 1 µg of oligo-(dT)₁₈ primer, 200 µM dNTPs, 5× RT buffer, 10 mM dithiothreitol, and 200 U of AMV reverse transcriptase (TaKaRa) at 42°C for 50 min after preheating at 70°C for 10 min to denature secondary structures, and terminated by heating at 70°C for 15 min. A two-step semi-quantitative RT-PCR method was used to measure gene expression of isolated β-1,3-glucanase genes according to Meadus (33) using an actin 2 (At3g18780) endogenous control to adjust the volume of each cDNA pool to give the same exponential phase PCR signal strength. Gene-specific primers used included *Glu-1* sense (5'-TCTACCA-TGGCAATGAATAATTTCCGAG-3') and antisense (5'-TGCC-TGCAGTTACCAGTCTTTCATCTTAT-3') and *Glu-2* sense (5'-CCCTCTCTCTCTTCTCTCATTTTCC-3') and antisense (5'-CAATAACCTGTAGGAACTCGCAC-3') for *Glu-1* and *Glu-2*, respectively. Cycling parameters were 94°C for 2 min; 25 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 90 s; and, finally, 72°C for 10 min. Both technical and biological repetitions were performed for all semi-quantitative RT-PCR experiments with RNAs from two independently treated fruit samples and two semi-quantitative RT-PCRs for at least one RNA for each treatment. The quantification was based on the level of product from an ethidium-bromide-stained gel using AlphaEaseFCTM software (version 4.0.0; Alpha Inotech Corporation, San Leandro, CA). Only band intensities that were >1.5-fold higher than the control (0 h in each individual experiment) were considered as inducible expression.

Sequence analysis. DNA and protein sequences were compared with the sequences deposited in GenBank using the BLAST program. N-terminal signal peptides were predicted using PSORT online version 6.4 software for plants (36). Cleavage of the signal peptide was predicted according to von Heijne (50). The consensus sequence for N-glycosylation (NX-S/T), the conserved signature for glycosyl hydrolases family 17, and the amino acid residues of the catalytic nucleophile and catalytic acid were identified according to Henrissat (18) and Hoj et al. (19).

RESULTS

Interaction between yeast and pathogens in vitro and in vivo. *C. laurentii* significantly inhibited spore germination and germ tube growth of *P. expansum* and *A. alternata* in PDB (Table 1). For untreated controls, the germination rate of *P. expansum*

and *A. alternata* reached 90.5 and 82.3% with a germ tube length of 105.8 and 91.2 µm, respectively. However, for treatments containing *C. laurentii*, only 40.2 and 25.2% of spores germinated, with germ tube length of 32.5 and 28.3 µm for *P. expansum* and *A. alternata*, respectively.

Growth of *C. laurentii* in the wounds of jujube fruit in all treatments was observed with prolonged storage time at 25 and 0°C (Fig. 1). At the beginning of the experiment (time 0), the population of the yeast was $\approx 7.2 \times 10^5$ CFU/wound, and increased to 1.3×10^7 CFU at 25°C and 5.1×10^6 CFU at 0°C. This corresponded to an 18.1- and 7.1-fold growth increase compared with the initial inoculum at 25 and 0°C, respectively. In addition, the amount of yeast in the wounds when the pathogens were present was significantly higher than in the absence of pathogens. These phenomena indicated that the presence of pathogens in the wound could stimulate an increase in yeast numbers. It also was clear that *C. laurentii* has adapted to low-temperature environments, because it thrived even at 0°C.

Effect of different concentrations of yeast on decay control. The effectiveness of *C. laurentii* against *P. expansum* and *A. alternata* in jujube fruit was positively correlated with concentrations of the yeast. At 1×10^7 CFU/ml, *C. laurentii* significantly ($P < 0.05$) reduced disease incidence and lesion diameter caused by both fungal pathogens compared with the control. Furthermore, better disease control was observed at inoculum concentrations of 1×10^8 than at 1×10^7 CFU/ml at both 25 and 0°C (Figs. 2 and 3). After storage at 25°C for 4 days, disease incidence in the control fruit reached 100% (with 11-mm lesion diameter), whereas a 78.7 and 20.7% change in disease incidence (with 8- and 5-mm lesion diameter) caused by *P. expansum* was found in the fruit treated with *C. laurentii* at 1×10^7 and 1×10^8 CFU/ml, respectively. A 60.3 and 3.3% change in disease incidence (with 5- and 3-mm lesion diameter) by *A. alternata* was observed in the same conditions (Fig. 2). Low temperature delayed disease development. The control fruit showed 100% disease incidence after storage at 0°C for 25 days, whereas the fruit treated by *C. laurentii* at 1×10^8 CFU/ml had only 13.3 and 23.3% disease incidence resulting from *P. expansum* and *A. alternata*, respectively (Fig. 3). In addition, lesion diameters caused by both diseases were reduced significantly ($P < 0.05$) by increasing concentration of *C. laurentii* at both 25 and 0°C (Figs. 2 and 3).

Changes in β-1,3-glucanase by wounding and yeast inoculation. Activity of β-1,3-glucanase in the control fruit changed slightly during the experiment periods; however, wounding stimulated an increase in β-1,3-glucanase activity between 24 and 48 h after treatment. β-1,3-Glucanase activity in the fruit wounded and inoculated with *C. laurentii* increased more markedly and was present at significantly higher levels compared with wounding alone or the untreated controls (Fig. 4A). Activity of β-1,3-glucanase in *C. laurentii*-treated fruit increased by more than 2.8- and 2.4-fold than the control and by 2.3- and 1.7-fold compared with the wounded, noninoculated fruit at 24 and 48 h, respectively, after treatment. In addition, β-1,3-glucanase activity was significantly enhanced with increased concentrations of the yeast and showed the peak value at 48 h in all treatments (Fig. 4B). Fruit dipped in a suspension of *C. laurentii* at 1×10^8 CFU/ml gave rise to the highest activity of β-1,3-glucanase among all

TABLE 1. Effect of *Cryptococcus laurentii* at 1×10^7 CFU/ml on spore germination and germ tube length of *Penicillium expansum* and *Alternaria alternata* in potato dextrose broth after 12 h of incubation at 25°C^a

Treatments	Spore germination (%)		Germ tube length (µm)	
	<i>P. expansum</i>	<i>A. alternata</i>	<i>P. expansum</i>	<i>A. alternata</i>
Control	90.5 ± 1.11 a	82.3 ± 0.52 a	105.8 ± 2.54 a	91.2 ± 2.13 a
<i>C. laurentii</i>	40.2 ± 0.15 b	25.2 ± 0.63 c	32.5 ± 0.29 d	28.3 ± 0.45 d

^a Means in a column followed by a different letter differ significantly at $P = 0.05$ by Duncan's multiple range tests, and ± data represent standard deviations of the means.

treatments, but activity of β -1,3-glucanase in the control fruit was lowest throughout the duration of the experiment.

Cloning β -1,3-glucanase genes from jujube fruit. Full-length cDNA sequences of two distinct β -1,3-glucanase genes were

obtained from jujube fruit 24 h after wounding and designated as *Glu-1* and *Glu-2* (GenBank accession nos. DQ012940 and DQ093571, respectively). The cDNA of *Glu-1* was composed of 1,265-bp nucleic acids with an 87-bp 5' untranslated region, a

Fig. 1. Population dynamics of *Cryptococcus laurentii* with and without fungal pathogens in the wounds of jujube fruit at **A and C**, 25°C and **B and D**, 0°C. Bars represent standard deviations of the means.

Fig. 2. Effects of different concentrations of *Cryptococcus laurentii* on disease incidence and lesion diameter caused by *Penicillium expansum* and *Alternaria alternata* in jujube fruit stored at 25°C after 4 days. The fruit were wounded and inoculated with *C. laurentii* at different concentrations of 1 × 10⁷ and 1 × 10⁸ CFU/ml. Bars represent standard deviations of the means. Values followed by different letter were significantly different according to Duncan's multiple range test at *P* < 0.05.

1,134-bp open reading frame (ORF), and a 41-bp 3' untranslated region terminating in a poly (A) tail. An N-terminal signal peptide consisting of 22 amino acids was identified from the encoding protein using PSORT software, indicating that the Glu-1 protein was likely to be transferred to the extracellular space. Cleavage of this signal peptide was predicted to yield a mature protein of ≈ 39 kDa with a calculated isoelectric point of 5.9. A consensus sequence for NX-S/T was located at amino acid positions 281 to 283 (N-E-S), indicating that Glu-1 may be a glycoprotein similar to other previously characterized β -1,3-glucanase proteins. The amino acid residues of the catalytic nucleophile and catalytic acid were glutamic acids at position 271 and 334. Glu-1 shared significant identity with sequences of identified β -1,3-glucanases from plants such as *Arabidopsis* (accession no. AAM67102, 77%),

rice (AP005446, 64%) potato (S31196, 49%), and tobacco (AB050628, 41%).

The cDNA of *Glu-2* contained a 70-bp 5' untranslated region, a 1,407-bp ORF, and a 7-bp 3' untranslated region terminating in a poly (A) tail. The ORF encoded a 468-amino-acid polypeptide with a predicted N-terminal signal peptide cleavage site at the 19th amino acid, indicating that Glu-2 also was likely to be an extracellular protein. The amino acid residues of the catalytic nucleophile and catalytic acid were glutamic acids at position 260 and 323, respectively. A protein database search showed that Glu-2 had significant identity to putative β -1,3-glucanase-like proteins from *Arabidopsis* (AAM66024, 71%), tobacco (AB032530, 70%), rice (XM468018, 56%), and maize (AY542797, 39%). Despite the similar cellular location and putative function, Glu-1 and

Fig. 3. Effects of different concentrations of *Cryptococcus laurentii* on disease incidence and lesion diameter caused by *Penicillium expansum* and *Alternaria alternata* in jujube fruit stored at 0°C after 25 days. The fruit were wounded and inoculated with *C. laurentii* at different concentrations of 1×10^7 and 1×10^8 CFU/ml. Bars represent standard deviations of the means. Values followed by different letter were significantly different according to Duncan's multiple range test at $P < 0.05$.

Fig. 4. Effects of wounding and *Cryptococcus laurentii* on β -1,3-glucanase activity in jujube fruit. **A**, Comparison of β -1,3-glucanase activity in fruit that were wounded alone, wounded and inoculated with *C. laurentii* at 10^8 CFU/ml, or unwounded and noninoculated as the control. **B**, Comparison of β -1,3-glucanase activity in jujube fruit dipped in suspensions of *C. laurentii* at different concentrations. Sterile water was used as the control. Bars represent standard deviations of the means.

Glu-2 shared only 43% identity at the amino acid level, suggesting that they may not be functionally redundant. Amino acid sequence comparisons showed that Glu-1 and Glu-2 were only 33 and 39% identical to fruit-specific peach β -1,3-glucanase (AF435089) (25), 36 and 40% identical to pathogen-induced soybean β -1,3-glucanase (U41323) (6), and 37 and 40% identical to fruit-specific banana β -1,3-glucanase (AF001523) (38), respectively. However, sequence alignment revealed that they all contained the conserved signature for glycosyl hydrolases family 17, as indicated by the conserved glutamic acid residues of the catalytic nucleophile and catalytic acid (Fig. 5).

Expression of β -1,3-glucanase genes in jujube fruit is induced by wounding and yeast inoculation. Analysis of gene expression further supported the distinct roles of *Glu-1* and *Glu-2* in response to wounding and yeast (Fig. 6). Both genes were constitutively expressed in jujube fruit and responded to wounding by increased expression compared with untreated controls ($1.88 \pm$

0.22 -, 2.25 ± 0.31 -, and 2.40 ± 0.21 -fold increases were observed for *Glu-1* and 1.39 ± 0.19 -, 1.65 ± 0.31 -, and 1.69 ± 0.20 -fold increases were observed for *Glu-2* at 12, 24, and 48 h after wounding, respectively). However, the genes differed in their expression pattern after yeast inoculation: *Glu-1* expression level was induced with increasing period after inoculation and showed a peak value at 48 h (2.79 ± 0.33 -, 3.99 ± 0.41 -, and 5.96 ± 0.32 -fold compared with the untreated control at 12, 24, and 48 h, respectively), whereas *Glu-2* expression after wounding and subsequent yeast inoculating was not different from that after wounding alone (1.54 ± 0.18 -, 1.41 ± 0.21 -, and 1.51 ± 0.19 -fold compared with the untreated control at 12, 24, and 48 h, respectively), suggesting that Glu-2 was unaffected by yeast inoculation. The actual relative band intensity values, which are well consistent with the images in Figure 6, revealed that *Glu-1* was highly induced by both wounding and antagonist, whereas *Glu-2* was broadly not responsive to yeast inoculation. Gene expression

Peach	1	MAKSNSSVGRGSPSMISIVFLLGLLMAS-FETTGAAQIGVCYGM LGDRLP PPSEVIALYKQN
Soybean	1	MAKYHLS-GKSSSITSIAFLFILLITNTGKAGA QSGVCYGRIGNNLPSPQEVVALFKQY
Banana	1	MAT-----KASLSIKGFALLVSVLVAVPTRVQ--SIGVCYGM LGNNLP PPSEVVS LYKSN
Glu-2	1	MAS-----ITFSLLLSLLFAFTDAGS--IGINYGRVANDLP TPSKVVELLKSQ
Glu-1	1	MNNFR---VFSLFVYVFLLELPPIASLTVQAF TGTGYGINYGRIADNIPSPDEVVTL LRAA
Peach	60	NIRRMRLYDPNQAALAAALKGSYIELMLGVPNDNLQSLASSQANANTWVONNVNRYG-NVR
Soybean	60	DFFRRMR YDPSQEVLEALRGSNIELLDIPNDNLQNLAFSQDNANKWLQDN IKNYANNVR
Banana	54	NIARMRLYDPNQAALQALRNSNIQVLLDVPRSDVQSLASNP SAAGDWIRRN VVAYWPSVS
Glu-2	47	GIDRVKLYD TDSVLTALANS GIGVVVALPNELLSSSTANDQSFADK WVQANISQYYPKTK
Glu-1	58	KIKNVRLTYDAEHSVLKAFSGTGLELVVGLP NGLLKDMSANADHAMEWVKENVQAF LPETH
Peach	119	FKYIAVGNEVKPS--DSYAQFLVPAMONIQNAISSAGLG--IKVSTAVDTGVLGNSEPPS
Soybean	120	FRYISVGNEVKPE--HSFAQFLVPAMONIQRAISNAGLGNQIKVSTAIETGALADSYPPS
Banana	114	FRYIAVGNE LIPG--SDLAQYILPAMNIYNALSSAGLQNKIKVSTAVDTGVLGTSYPPS
Glu-2	107	IEAIAVGNEVFVDP-NNTTKFLVPAMKNIHTSLSKYNLND SIKISSPIALSALQSSYPSS
Glu-1	118	ICGIAVGNEVLGGS DYELWGALLGAVKNICNAVKKLNLDDVVQITTAHSQAVFSNSYPPS
Peach	175	KGEFKSEYG-ALLNPIIRFLVNNRSPLLVNLYPYFSYSSNTHDIRLDYALFTAPS VVVQD
Soybean	178	MGSERSDYRTAYLDGVIRHLVNNNTPLLNVNYPYFAYINDERNISLDYALFRSPSVVVQD
Banana	172	AGAFSSAAQ-AYLSPIVQFLASNGAPLLNVNYPYFSYTGNGQISLPYALFTASGVVVQD
Glu-2	166	SGSEKTELVGPFV KPMLDLLRQTSSYLMVNAYPFFAYIDNSDTIPLAYALFQT NAGTVDS
Glu-1	178	SCTFR-DNVVQYMKPLLEFFAQVGS PFCLNAYPFFAYMSDPENIDIN YALFKSTQGIYDP
Peach	234	GQ-RGYRNLFDAILDAVYAAL EKAGGGSLEIVISESGWPSAGG---TATTIDNARTYNAN
Soybean	238	GS-LGYRNLFDAMVD AVYAAL EKAGGGSVSI VVSESGWPS SGG---TATSLDNARTYNTN
Banana	231	GR-FSYQNLFD AIVDAVFAALERVGGANVAVVVSESGWPSAGGG--AEASTSNARTYNQN
Glu-2	226	GNGLRCNSLLEAQIDAVYAAMNALGYNDVKLVVTTETGWPSKGDENEIGATQANAAS YNGN
Glu-1	237	KTDLHYDNMLDAQIDAAYAALENAGYKKMEVVVTTETGWASRGDENESAATVNNARTYNYN
Peach	290	LIQHVKGK--TPRKP-GRAIET YIFAMFDENRKN-PELEKHWGLFSPSKQPKYPINFN--
Soybean	294	LVRNVKQG--TPKRPAGRPLET YVFAFNENHKQ-PEYEKFWGVFLPNKQPKYSINLN--
Banana	288	LIRHVGGG--TPRRP-GKEIEAYIFEMFNENOKA-GGIEQNFGLFYPNKQPVYQISF--
Glu-2	286	LVRRLVTGSGTPLRP-QDPLNVFLFA FNFENEEKSGPTSERNYGLFYPSEEKVYDIPLTKA
Glu-1	297	LRKRLAKKKGTPLRP-KNVVKA YIFAAFNEDLKPGATSERNEGLFKADGTIAYDIGF HGL
Peach		-----
Soybean		-----
Banana		-----
Glu-2	345	EVNGGVQSTTPANGTNSTTEISPAGEVSKTSVGQTWCVANGNVGEEKLTALDYACGEGG
Glu-1	356	DSSSAKSLLLYLKFTLVYKMKDW-----
Peach		-----
Soybean		-----
Banana		-----
Glu-2	405	ADCRPIQEGSTCYDPNSLVAHASYAFNSYYQK KARLIGSCDFGGAAYVATQPPKFGQCEP
Glu-1		-----
Peach		----
Soybean		----
Banana		----
Glu-2	465	PTGY
Glu-1		----

Fig. 5. Comparisons of the jujube Glu-1- and Glu-2-deduced amino acid sequences with homologous sequences from other plants as indicated. Identical residues and conserved amino acids have been shaded in black and in gray, respectively. Dashes (–) indicate the absence of residues. The predicted N-terminal signal peptides of Glu-1 and Glu-2 are boxed, and the conserved signature for glycosyl hydrolases family 17 are underlined. The glutamic acid residues of the catalytic nucleophile and catalytic acid are indicated by asterisks. The putative consensus sequence for N-glycosylation (N-E-S) in Glu-1 is indicated by the dotted line.

analysis of jujube fruit that was incubated in increasing concentrations (from 10^6 to 10^8 CFU/ml) of yeast culture showed that *Glu-1* was significantly stimulated (1.76 ± 0.22 -, 2.18 ± 0.18 -, and 2.97 ± 0.31 -fold compared with the untreated control at yeast concentration of 10^6 to 10^8 CFU/ml for *Glu-1* and 1.26 ± 0.20 -, 1.04 ± 0.13 -, and 1.28 ± 0.31 -fold for *Glu-2*), whereas *Glu-2* remained unchanged regardless of yeast concentration (Fig. 7). These results suggested that, in contrast to *Glu-2*, *Glu-1* is yeast responsive and may play a role in defense response of jujube fruit against fungal infection during the postharvest period.

DISCUSSION

In our previous studies, *C. laurentii* was isolated from the surface of apple fruit and evaluated to be effective in controlling postharvest diseases in jujube fruit (39,45). In this study, we further found that *C. laurentii* could significantly limit spore germination and germ tube growth of *P. expansum* and *A. alternata* in PDB (Table 1), and showed a rapid increase in population dynamics in the wounds of jujube fruit, particularly when pathogens were present (Fig. 1). Meanwhile, *C. laurentii* could propagate rapidly in the wounds of jujube fruit 48 h after inoculation (Fig. 1), resulting in an effective decrease in disease incidence and lesion diameter caused by *P. expansum* and *A. alternata* (Figs. 2 and 3). In general, the mode of action of microbial biocontrol agents may involve nutrient competition, site exclusion, direct parasitism, and perhaps induced resistance (12). However, antagonist-pathogen interaction has been considered to play a major role in their biological activities (8). Some results indicated that the difference in biocontrol ability of yeasts against fungal pathogens might be relative to attachment to fungal hyphae (14, 52). Wisniewski et al. (52) found that yeast cells produce lytic enzymes that can enhance the attachment to hyphae of pathogens. Our previous results indicated that *P. membranefaciens* had a stronger capability of attaching to the fungal hyphae of *P. expansum* than *Cryptococcus albidus* did both on apple juice agar plates and in apple wounds by light microscopy and scanning electron microscopy observations, because the differences might be attributed to cell membrane surface proteins and protein signal recognition between yeast cells and pathogen hyphae (3).

Induction of disease resistance in a number of crops following treatment with microbial agents has been demonstrated and provides protection against a wide range of pathogens (27,49). In harvested commodities, the induction of disease resistance by microbial antagonists has been suggested in recent years (10,20). Protection of the fruit from the invasion of fungal pathogens appears due to involvement of a highly coordinated biochemical and structural defense system that helps ward off the spread of

pathogens (28,41). The onset of resistance often has been correlated with the accumulation of defense-related enzymes, such as chitinases, β -1,3-glucanases, phenylalanine ammonia lyases, and peroxidases (12,55). Several in vitro experiments have indicated that β -1,3-glucanases and chitinases were able to partially degrade the cell walls and to inhibit mycelial growth or spore germination of some pathogenic fungi (1,28). Furthermore, the antifungal effects were synergistically enhanced when both enzymes were present. In jujube fruit, β -1,3-glucanase activity was significantly induced by *C. laurentii* 24 h after inoculation (Fig. 4). This result is consistent with the temporal and spatial induction of defense-related enzymes and likely concomitantly with induced resistance (43). It also documents that jujube fruit were capable of responding to wounding and yeast, as seen in other crops (10,15,22). Additionally, the induction of resistance was positively correlated with the concentration of biocontrol agents. Enhancing concentration of *C. laurentii* can induce an increase in β -1,3-glucanase activity (Fig. 4) and expression of the genes (Fig. 7), resulting in effective control of decay by *P. expansum* and *A. alternata* in jujube fruit (Figs. 2 and 3). We previously have demonstrated that yeast treatment induced synthesis of antioxidant enzymes and variety of total protein,

Fig. 7. Semi-quantitative reverse-transcription polymerase chain reaction analysis of *Glu-1* and *Glu-2* expression in response to different concentrations of *Cryptococcus laurentii* in jujube fruit. Total RNAs were obtained from jujube fruit treated with *C. laurentii* at 10^6 to 10^8 CFU/ml for 48 h. The housekeeping gene actin 2 (At3g18780) was used as a control for normalizing mRNA quantity. Relative expression ratio of *Glu-1* and *Glu-2* in each yeast-inoculated fruit compared with that in untreated fruit is shown below each band.

Fig. 6. Semi-quantitative reverse-transcription polymerase chain reaction analysis of *Glu-1* and *Glu-2* expression in response to wounding and *Cryptococcus laurentii* in jujube fruit over time. Total RNAs were obtained from jujube fruit wounded by a sterile nail and subsequently inoculated with 20 μ l of *C. laurentii* at 10^8 CFU/ml at indicated hours. The housekeeping gene actin 2 (At3g18780) was used as a control for normalizing mRNA quantity. Relative expression ratio of *Glu-1* and *Glu-2* in each wound-treated or yeast-inoculated fruit compared with that in untreated fruit is shown below each band.

which may play a role in the resistance against blue mould rot in sweet cherry (4). This increased H_2O_2 has been proposed to be a signal leading to hypersensitive cell death, and activates many tolerance-related genes against pathogen attack (26).

Generally, β -1,3-glucanase genes in plant species exist in multiple isoforms (16). β -1,3-Glucanases from tobacco can be divided into three classes based on structural similarity: class I contains vacuolar (intracellular) β -1,3-glucanases with a basic isoelectric point (pI) and abundant mRNA transcripts in roots of healthy plants (42); class II contains acidic secreted β -1,3-glucanases which are induced after infection by pathogen or elicitors (30) and appear to be localized in the extracellular compartment; and class III contains tobacco extracellular PR β -1,3-glucanases which are induced by *Tobacco mosaic virus* infection and are more closely related to class II in structure, regulation, and function (37). Sequence analysis revealed that Glu-1 and Glu-2 are acidic secreted β -1,3-glucanases, suggesting that they belonged to class II. Based on the observed spatial and temporal regulation of expression, β -1,3-endoglucanases appear to participate in different biological functions, such as plant reproductive biology and PR defense mechanisms against fungal attack (42). β -Glucanases also may be involved in other processes, such as in response to wounding and abiotic stress (35,38,56). Expression of Glu-1 and Glu-2 was stimulated in jujube fruit 12 to 48 h after wounding (Fig. 6), suggesting that the timing and expression level of β -1,3-glucanase genes may be an important aspect in defense responses (53).

Glu-1 was highly induced by both wounding and inoculation with *C. laurentii*, particularly at 24 and 48 h, whereas *Glu-2* was not responsive (Fig. 6). These results are in agreement with Wu and Bradford (53), who detected expression of β -1,3-glucanase (*GluB*) in tomato leaves in response to wounding and methyl jasmonate treatment after 12 h. Because β -1,3-glucanase was strongly induced by the biocontrol agent in jujube fruit, the localized expression of β -1,3-glucanase that would be in direct contact with the biotic environment might constitute an effective barrier to pathogen entry into the fruit. Additional support for this conclusion was derived from the study of Wu et al. (54) in tomato. There is compelling evidence that β -1,3-glucanase can act directly by degrading the pathogen cell wall or indirectly by releasing oligosaccharide elicitors of defense reaction, both of which are potential defense mechanisms against fungal infection (6,21). These observations supported the notion that different members of β -1,3-glucanase probably are involved in various biological functions in plant defense response against a wide variety of pathogens (30,37,48). Our findings open out a mechanistic framework for the functions of β -1,3-glucanase in fruit defense responses.

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