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cDNA cloning, expression, protein purification, and characterization of a novel glyoxal oxidase related gene from *Vitis pseudoreticulata*

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Abstract

To study the defense mechanism of the resistance to the disease of Chinese wild *Vitis* species and offer powerful bases for the molecular breeding of highly disease-resistant grape cultivars, using mRNA differential display reverse transcript-PCR (DDRT-PCR) and RACE, a full-length cDNA was isolated from Chinese wild *Vitis pseudoreticulata* clone Baihe-35-1 inoculated with *Uncinula necator* by pressing infected leaves under natural field conditions. The cDNA designated as GLOXrg is 1708 bp in length with an open reading frame of 1572 bp encoding 523 amino acids, containing the conserved domain, glyoxaloxid N domain (pfam07250). No homologous nucleotide sequence was found, but the deduced amino acid sequence of GLOXrg shows 74 % identity with a putative protein from *Arabidopsis thaliana* (accession No. CAB88357). GLOXrg was cloned into pGEX-4T-1 vector. The recombinant vector expressed an about 83-kDa GST-GLOXrg fusion protein as insoluble inclusion bodies in *Escherichia coli* BL21. Fusion protein GST-GLOXrg was isolated and used to raise the polyclonal anti-(GST-GLOXrg) in rabbits. Western blot analysis showed a high titer (10 000) and the high specificity of the polyclonal anti-(GST-GLOXrg). This is the first report on the cloning of the full-length GLOXrg cDNA from the plant except *Arabidopsis thaliana*. Through the differential expression, GLOXrg probably plays a crucial role in the resistance to *Uncinula necator* of Chinese wild *Vitis* species. An optimized system on prokaryotic expression, protein purification and preparation of the polyclonal antibodies of GLOXrg was also established.

Additional key words: Chinese wild *Vitis* species, fusion protein expression, gene cloning, mRNA differential display, polyclonal antibodies.

Introduction

Powdery mildew, caused by *Uncinula necator* (Schw.) Burr., is the most ubiquitously damaging fungal disease of grapevines worldwide, reducing yield, vine growth and vigor, and fruit quality (Pool *et al*. 1984, Gadoury *et al*. 2001). Control of powdery mildew on grapevine is currently achieved by the widespread application of fungicides such as sulphur and, more recently, systemic de-methylation inhibitors. Utilization of host plant resistance through breeding would be an effective and economical strategy to control the disease. Sources of disease-resistant grape germplasm are needed as the foundation for such a breeding program (Lenne and Wood 1991).

Most of European grape cultivars with fine quality and high yield are susceptible to powdery mildew, but *V. labrusca* and Chinese wild *Vitis* species are resistant to it. China is one of the major centers of origin of *Vitis* species (He *et al*. 1991). In addition to their potential as sources of disease resistance, Chinese wild *Vitis* species do not have the foxy flavor that limits the use of some of American native grapes in breeding programs (Alleweldt and Possingham 1988). However, utilization of the

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Abbreviations: GLOXrg - glyoxaloxidase related gene, GLOX - glyoxaloxidase, GST - glutathione S-transferase, IPTG - isopropyl-β-D-thiogalactoside, DDRT-PCR - differential display reverse transcription-PCR.

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identified germplasm for breeding new cultivars through conventional genetic breeding may take a significantly long time because of possible association of the resistant trait with undesired traits. Alternative strategies, involving chemical or genetic manipulation of host defense mechanisms, have been shown to increase the resistance of a number of crop species to fungal pathogens (Hain *et al*. 1993, Jach *et al*. 1995, Jongedijk *et al*. 1995). Therefore, understanding the host defense mechanism and identifying key genes in the resistant germplasm should provide valuable information and foundational resources for the timely and efficient molecular breeding of highly resistant cultivars.

Plants respond to environmental stimuli and pathogen attack via the induction of a number of different defense mechanisms. These include the deposition of mechanical barriers such as sugars and hydroxyproline-rich glycoproteins within cell walls to limit invasion by fungal hyphae (Aist 1976, Showalter *et al*. 1985) and the synthesis of small secondary stress metabolites, such as phytoalexins, which have been shown to be fungitoxic (Hain *et al*. 1993). The production of active oxygen species within cells has also been linked with plant defense processes (Brisson *et al*. 1994). There are many researches on the resistance to *Uncinula necator* of grapevine. A number of pathogenesis-related (PR) cDNA clones from *V. vinifera* cultivars Sultana and Cabernet Sauvignon infected with powdery mildew, such as chitinases (PR-2), beta-1, 3-glucanases (PR-3), and thaumatin-like (TL) proteins (PR-5), were reported (Jacobs and Robinson 1999). The research of expression of chitinase genes in *V. vinifera* cultivar Ugni Blanc induced by *Uncinula necator* was also reported (Robert *et al*. 2002). In addition, a number of resistance gene analogs linked to a powdery mildew resistance locus in *V. vinifera* were isolated (Donald *et al*. 2002). Moreover, transgenic grapevine plants expressing a rice chitinase have also been showed to enhance resistance to fungal pathogens (Yamamoto *et al*. 2000). So far, the researches on the cloning of genes related to the resistance to *Uncinula necator* in Chinese wild *Vitis* species are not still reported.

Chinese wild *Vitis pseudoreticulata* clone Baihe-35-1, which originated from the Baihe county, Shaanxi province of China, is highly resistant to most of pathogens of *V. vinifera* including *Uncinula necator* (Wang *et al*. 1995, Wang and He 1997). In this research, using DDRT-PCR and RACE, the full-length cDNA sequence of glyoxaloxidase related gene (GLOXrg) from Chinese wild *Vitis* species was firstly cloned from the plant except *Arabidopsis thaliana*, based upon GenBank data. We also described the expression in *Escherichia coli* of GLOXrg in fusion with GST of *Schistosoma japonicurn*, the purification of the resulting chimeric protein GST-GLOXrg and preparation of the polyclonal anti-(GST-GLOXrg) serum.

Materials and methods

Plants and treatments: Chinese wild species *Vitis pseudoreticulata* W.T. Wang clone Baihe-35-1 highly resistant to powdery mildew and *V. adstricta* Hance clone Taishan-2 highly susceptible to powdery mildew maintained in the grape germplasm resources orchard, Northwest A&F University, Yangling Shaanxi, P.R. China, were used for the present study. The powdery mildew inoculation was carried out under natural field conditions by pressing infected leaves with *Uncinula necator* of Taishan-2 against the uninfected leaves of Baihe-35-1 pre-sprayed with sterile water from 08:00 to 10:00 on 12 August, 2002. The inoculated leaves were immediately covered with paper bags for infection, and the leaves only sprayed with sterile water were used as control.

General DNA techniques: All enzymes for DNA manipulations were purchased from TaKaRa biotechnological Co. (Dalian, China) and applied according to manufacturer's instructions.

Escherichia coli DH5a (*Amersham Biosciences*, Piscataway, NJ, USA) strains were used for cloning plasmid constructions. *Escherichia coli* BL21 (*Amersham Biosciences*) strains were used for expression plasmid constructions.

Plasmid DNA was isolated using the plasmid extraction kit (*Tiangen*, Beijing, China), DNA fragments were isolated using DNA agarose gel cleanup kit (*Tiangen*).

Total RNA isolation and DDRT-PCR: Total RNA was isolated from the grape leave samples 0, 1, 3, 5, 7, and 9 d after treatment by the method of SDS/phenol with some modifications (Zhang *et al*. 2003). mRNA differential display was performed as described by Liang and Pardee (1992). In reverse transcription reactions, the oligonucleotide was used as anchor primer: 5'-TTTTTTTTTTTAC-3' (T_{11}AC). The reaction conditions for reverse transcription (0.02 cm^3) is 1.0 μg total RNA, 6.0 mM anchor primer T_{11}AC, 0.25 μM dNTPs, 20 U RNasin, 5× transcription buffer 0.004 cm^3, and 200 U M-MMLV transcriptase (*Promega*, Madison, WI, USA). Reactions were incubated at 70 °C for 5 min, cooled in ice and 20 U RNasin, 200 U M-MMLV transcriptase added, then incubated for 60 min at 37 °C and 95 °C for 5 min. PCR reactions (0.025 cm^3) were performed with 0.001cm^3 RT reaction first strand, 2.5 μM anchor primer T_{11}AC, 2.5 μM arbitrary primer (S421~S440, *Sangon*, Shanghai, China), 0.25 mM dNTPs, 1.0 U *Taq* DNA polymerase. PCR program as following: 94 °C for 1 min, 40 cycles of 94 °C for 30 s，40 °C for 2 min，72 °C for 30 s and a final extension step 72 °C for 10 min. 0.0045 cm^3 product of DDRT-PCR was

separated on a 6 % polyacrylamide sequencing gel for 3.5 h at 2000 V. To detect false-positive, both reverse transcription and PCR reactions were carried out in duplicate for each sample. The results of DDRT-PCR were analyzed by a silver-stain method with the following modifications (Bassam *et al*. 1991). The gel was fixed in 10 % (v/v) acetic acid for 30 min, rinsing with H_2O and incubated with solution A [1 g dm^{-3} $AgNO_3$, 1.5 cm^3 dm^{-3} 37 % (v/v) formaldehyde] at 37 °C for 30 min, rinsed 2 - 3 times with H_2O, developed in solution B [30 g dm^{-3} Na_2CO_3, 1.5 cm^3 dm^{-3} 37 % formaldehyde, 2 mg dm^{-3} $Na_2S_2O_3 \cdot 5H_2O$] and fixed in solution C [10 % (v/v) acetic acid]. The gel was then washed twice with H_2O and air-dried at room temperature. The cDNA bands of interest were excised from the dried gel with a scalpel. Gel slices were heated in 0.03 cm^3 distilled water at boiling temperature for 10 min to elute cDNAs and used directly for PCR re-amplification with the annealing temperature 42 °C. The products of re-amplification were cloned into *pGEM-T Easy* vector (*Promega*), followed by sequencing (*TaKaRa Biotechnology Co.*).

5' RACE of specific cDNA fragment: In order to obtain the full-length cDNA sequence of a novel gene, 5' RACE was carried out. The gene specific primer GSP1: 5'-TACTCGCAGAATGAACCCAGTCCAGC-3' for 5' RACE was designed based on the sequence of the specific cDNA fragment T_{11}AC/B0313-307 containing the 3' end of the novel gene. Total RNA of Baihe-35-1 leaves inoculated with *Uncinula necator* after 7 d was isolated as described above and RACE was performed according to the manufacturer's instructions (*BD SMART*™ RACE cDNA amplification kit). The 5' RACE products was separated by the 1.2 % agarose gel electrophoresis and purified with DNA agarose gel cleanup kit (*Tiangen*). The 5' RACE fragment was cloned into pGEM-T Easy vector (*Promega*), and sequenced by *Takara Biotechnology Co.*

Sequence analysis of the full-length cDNA: The full-length cDNA sequence with 1708 bp was obtained through overlapping sequence of 5' RACE fragment and the specific T_{11}AC/B0313-307 fragment containing the 3' end. Homologous sequences searches were performed by blastn and blastx (http://www.nebi.ntm.nih.gov/blast). Motif was scanned using *Motif Scan* in a *Protein Sequence* (http://myhits.isb-sib.ch/cgi-bin/motif_scan). Searches of conserved domain were performed by CD-search program (http://www.ncbi.nlm.nih.gov/structure/cdd/wrpsb.cgi).

Construction of expression plasmid: With 5' RACE cDNA as template, cDNA of the novel gene was synthesized by polymerase chain reaction. Two oligonucleotides were used as primers: *1*) 5'-GCG GAT CCA TGA TCT TGG ATG CCG CC-3', which contains the nucleotide sequence located at the 5' end of the protease gene, and a *Bam*HI site (underlined) introduced in order to facilitate the following cloning steps, and *2*) 5'-GCG TCG ACT TAT GGG ACC AAT TGG AC-3', which contains the sequence downstream of the protease gene, including the *Sal*I site (underlined). PCR reaction system is: 0.0025 cm^3 10× buffer, 0.25 mM dNTPs, 1.5 μM primer (*1*), 1.5 μM primer (*2*), 1.0 U *Taq* DNA polymerase, 0.0025 cm^3 5' RACE cDNA, 0.0163 cm^3 ddH_2O. PCR program as following: After 5 min of incubation at 94 °C, the following 35 cycles were 94 °C for 30 s, 60 °C for 30 s, 72 °C for 2 min. Program was terminated with a 5-min incubation at 72 °C. The obtained material was analyzed on 1 % agarose gel, and then cloned into the pGEM-T Easy vector plasmids of the obtained colonies were isolated, and subjected to restriction endonuclease analysis, using *Bam*HI and *Sal*I. Positive clones were sequenced by *Takara Biotechnology Co*. This GLOXrg fragment was obtained by restriction endonuclease digestion of the recombinant pGEM-GLOXrg with *Bam*HI and *Sal*I and then cloned into pGEX-4T-1 (*Amersham Biosciences*), previously digested using the same enzymes mentioned above. The expression vector pGEX-4T-1 under the control of the tac promoter contains the GST gene (Smith and Johnson 1988). *Escherichia coli* BL21 competent cells were transformed for induced expression of GST-GLOXrg fusion protein. The recombinant vector pGEX-GLOXrg was identified by restriction endonuclease digestion.

Protein expression: Freshly prepared *Escherichia coli* culture bearing the appropriate plasmid was grown at 37 °C up to an absorbance at 600 nm of 0.8 - 1.0, in 20 cm^3 of Luria-Broth medium containing 0.1 % (m/v) ampicillin. Induction with 1 mM isopropyl-β-D-thio-galactoside (IPTG) was performed for 5 h, and cells were harvested by centrifugation at 10 000 *g* for 1 min, at 4 °C. After removal of the supernatant, cells were cleaved with *SuperE* lysis solution according to the manufacturer's instructions (*Biodee Biotechnology Co.*, Beijing, China). The supernate and sediment were boiled respectively in the loading buffer [50 mM Tris-HCl, pH 6.8, 2 % (m/v) sodium dodecyl sulfate (SDS), 1 % (m/v) bromophenol blue, 10 % (v/v) glycerol, 100 mM dithiothreitol (DTT)] for 3 - 5 min, and then analyzed by 12 % sodium dodecylsulfate-polyacrylamide gel electro-phoresis (SDS-PAGE). The culture was then shifted to the following processes in order to increase the proportion of soluble GST-GLOXrg fusion protein since the majority of the recombinant protein was found in inclusion bodies. Freshly prepared *Escherichia coli* culture was induced with IPTG of different concentrations (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, and 1.0 mM) at different temperature (20, 25, 30, and 37 °C) for different times (1, 2, 3, 4, 5, 6, and 7 h) and then analyzed by 12 % SDS-PAGE.

Protein purification: Cells were harvested by centri-fugation of the induced culture at 10 000 *g* for 1 min, at 4 °C, and then cleaved with *SuperE* lysis solution after

removal of the supernatant. Inclusion bodies containing the GST-GLOXrg fusion protein were centrifuged, washed for three times with 1 % (v/v) *TritonX-100* and 1 M guanidine hydrochloride respectively, and then dissolved in the solution containing 6 M guanidine hydrochloride, 50 mM Tris-HCl (pH 8.3), 5 mM DTT and 1 mM EDTA. The solution was then dialyzed against the buffer solution [10 mM Tris-HCl ,pH 8.3, 5 mM NaCl, 3 mM EDTA, 1 mM DTT, 5 % (m/v) saccharose, 20 % (v/v) glycerol] with the concentration grades of guanidine hydrochloride (2, 1, 0.5, 0 M). After centrifugation of the dialyzed solution, the expressed GST-GLOXrg fusion protein was isolated by affinity chromatography on glutathione-Sepharose according to the manufacturer's instructions (*Amersham Biosciences*). The purified protein was tested by 12 % SDS-PAGE.

Preparation of polyclonal antibody: Concentration of the purified GST-GLOXrg fusion protein was tested by the Bradford assay (Bradford 1976). The purified GST-GLOXrg fusion protein was treated with 9 M urea containing 0.1 M DTT (Rothel *et al*. 1997). The protein samples (≈700 μg) were separated by 12 % SDS-PAGE. The protein bands were visualized after being stained with 0.25 M KCl solution precooled. The recombinant GST-GLOXrg band was excised, triturated with liquefied N_2 and then mixed with 1 cm^3 PBS and 1 cm^3 Freund's complete adjuvant (*Sigma*, St. Louis, MO, USA). The mixture was utilized for intramuscular injections into New Zealand white rabbits. Polyclonal antiserum to GST-GLOXrg was raised in New Zealand white rabbits by three subcutaneous injections at 1-week intervals with ≈700 μg of GST-GLOXrg in Freund's incomplete adjuvant. Antiserum was collected after 4 weeks and stored at 4 °C in the presence of sodium azide (0.02 % final concentration) for the immunological studies.

Western blot analysis: Proteins were separated by 12 % SDS-PAGE and then transferred to nitrocellulose membrane (*Sigma*). The membrane was cut into strips, and then blocked with blocking buffer TBS-TM [15% (m/v) skim milk, 0.9 % (m/v) NaCl, 100 mM Tris-HCl (pH7.5), 0.1 % (v/v) *Tween-20*] at room temperature for 1 h, and then incubated with 1:10 000 and 1:100 000 dilutions of antiserum in TBS-TM at room temperature for 1 h. After being washed four times (15 min per time) with TBS-*Tween* buffer [100 mM Tris-HCl (pH 7.5), 0.9 % NaCl, 0.1 % (v/v) *Tween-20*], the strips were incubated with 1:500 dilution of Goat anti-rabbit IgG-AP (*SABC*, Luoyang, China) in TBS-TM at room temperature for 1 h and then washed four times with TBS-*Tween*. Antibody binding was visualized after being stained with *BCIP/NBT* kit (*SABC*).

Results

All the RT-PCR reactions were carried out and analyzed in duplicate in order to minimize spurious results. The cDNA fragment T_{11}AC/B0313-307 (accession No. DT725415), amplified with the anchor primer T_{11}AC and the random primer B0313 5'-GAT CAA GTC C-3', was expressed more strongly in leaves inoculated with *Uncinula necator* for 1, 3, 5, 7, and 9 d than in control leaves of Baihe-35-1 from all primer combinations (Fig. 1). Sequence analysis revealed that this fragment was 307 bp in length and contained a stop code TAA, a polyA tail and 120 bp 3' UTR.

5' RACE was employed to obtain the full-length cDNA sequence. A total of 0.006 cm^3 aliquots of the amplification products were separated on 1.2 % agarose gel after 5' RACE. 5' RACE product was about 1500 bp and then cloned into *pGEM-T Easy* vector, transformed into *E. coli* strain DH5α. The positive clone, charactered by blue/white screening and *Eco*R I digestion, was sequenced by *Takara Biotechnology* Co. It was actually 1480 bp in length.

One full-length cDNA with 1708 bp was obtained because of 79 bp overlapping sequence of 5' RACE fragment and T_{11}AC/B0313-307 fragment containing the 3' end, which is designated as GLOXrg (glyoxaloxidase related gene). The full-length cDNA sequence has been submitted into *GenBank* (accession No. DQ201181). GLOXrg contains an 1572 bp open reading frame that encodes 523 amino acids, and contains 5' UTR and 3' UTR and a polyA tail (Fig. 2). No homologous nucleotide sequence was found, but the deduced amino acid sequence of GLOXrg had 74 % identities to a putative protein from *Arabidopsis thaliana* (similar to

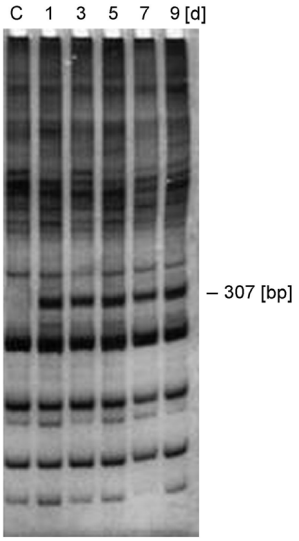

Fig. 1. The mRNA differential display of GLOXrg during attack of *Uncinula necator* (primers are T_{11}AC and B0313). GLOXrg was expressed more strongly in leaves inoculated with *Uncinula necator* for 1 d, 3 d, 5 d, 7 d and 9 d than in control leaves of Baihe-35-1 (C).

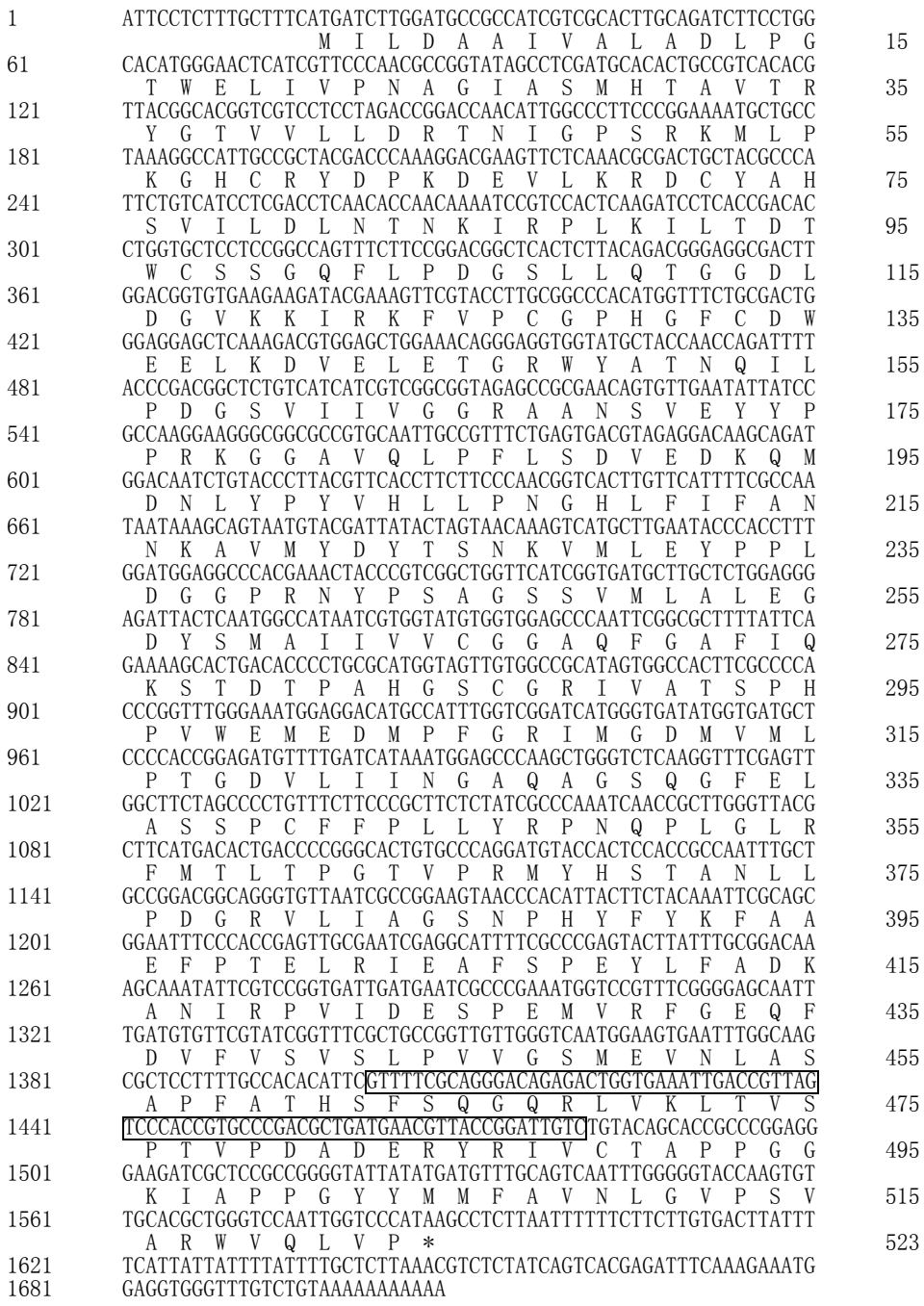

Fig. 2. Analysis of nucleotide sequence (*first line*) and deduced amino acid sequence from GLOXrg cDNA. Sequence in frame is the overlapping region between 5' RACE fragment and T11AC/B0313-307 fragment, underlined sequence in frame is the specific primer GSP1. GLOXrg cDNA contains the 1572 bp open reading frame that encodes 523 amino acids, 5' UTR, 3' UTR and a polyA tail. Nucleotide sequence data are available in the *GenBank* databases under the accession number DQ201181.

GLOX; accession No. CAB88357). No motif was found through *Motif Scan* in a *Protein Sequence*. Additionally, glyoxaloxid N, conserved domain, was found by CD-Search program.

GLOXrg cDNA was synthesized by PCR with 5' RACE cDNA as template. The amplified products were cloned into the *pGEM-T Easy* vector. The recombinant plasmid pGEM-GLOXrg was analyzed by digestion of restriction endonucleases *Bam*HI and *Sal*I. Positive clones were sequenced. The sequencing revealed the obtained sequence (1572bp) was identical to the open reading frame of GLOXrg cloned from *Vitis pseudo-*

reticulata Baihe-35-1. After GLOXrg was cloned into expression vector pGEX-4T-1, the recombinant plasmid pGEX-GLOXrg was digested by the restriction endonucleases *Bam*HI and *Sal*I. Agarose gel electrophoresis of the digestion revealed the correct construction of the recombinant plasmid pGEX-GLOXrg.

GLOXrg was cloned as fusion with GST, expressed, and analyzed by SDS-PAGE. Coomassie blue staining revealed a band of about 83 kDa for GST-GLOXrg (predicted molecular mass is 57 kDa plus 26 kDa for GST) (Fig. 3). The yield of the fusion protein accounted for 21 % of the total bacterial protein (analyzed with *Band Scan 4* program). The expression conditions (concentrations of IPTG, the induced time and the temperatures) were optimized, but the fusion protein was still expressed as inclusion bodies.

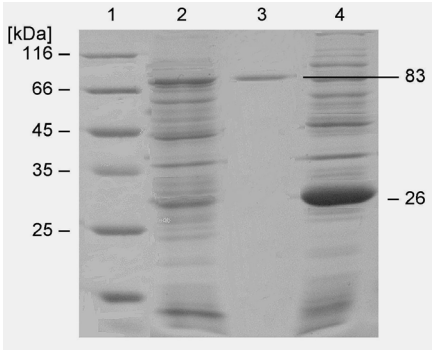

Fig. 3. Schematics of SDS-PAGE of purified GST-GLOXrg fusion protein. *Lane 1* - protein marker, *lane 2* - the protein from inclusion bodies of the recombinant plasmid pGEX-GLOXrg induced by 0.1 mM IPTG at 37 °C for 5 h, *lane 3* - purified GST-GLOXrg fusion protein, *lane 4* - the total protein of the empty vector pGEX-4T-1 induced by 0.1mM IPTG at 37 °C for 5 h.

Inclusion bodies were dissolved, and then dialyzed to renature. GST-GLOXrg fusion protein was isolated by affinity chromatography on glutathione-sepharose according to the manufacturer's instructions (Fig. 3).

To determine whether antiserum has the high specificity to the GST-GLOXrg fusion protein, the total protein induced was probed with 1:10 000 and 1:100 000 dilutions of antiserum in the Western blot analysis. The results showed an obvious protein band of 83 kDa closely correlated with the deduced molecular mass of GST-GLOXrg fusion protein (Fig. 4). These results demonstrated the specificity of generated antibodies. The appropriate dilution of antiserum for the further immunological studies was 1:10 000.

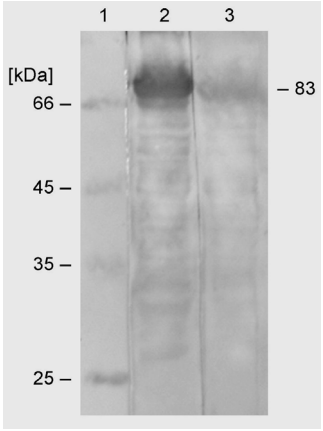

Fig. 4. Detection of Western blotting of the GST-GLOXrg fusion protein with rabbit anti-(GST-GLOXrg) serum. *Lane 1* - protein marker, *lane 2* - Western blotting with 1:10 000 dilution of antiserum, *lane 3* - Western blotting with 1:100 000 dilution of antiserum.

Discussion

The existence of multiple defense strategies and complex signalling networks of the plants leads to an enhanced defense capacity (Rea et al. 2002). The establishment of defense mechanism requires the presence or accumulation of H_2O_2 (Sutherland 1991, Mehdy 1994, Hammond-Kosack and Jones 1996). In particular, H_2O_2 behaves as a direct cytotoxic compound against pathogens and as a second messenger in the activation of defense genes (Lamb and Dixon 1997). Moreover, this compound is involved in systemic acquired resistance and acts synergistically with NO in the induction of hypersensitive cell death (Delledonne *et al*. 1998). As a substrate of the peroxidases, H_2O_2 has been implicated in the oxidative cross-linking of apoplastic structural proteins as well as in lignin and suberin polymerization (Rea *et al*. 2002). These events strengthen the plant cell wall after mechanical damage or pathogen challenge and make it less susceptible to the action of microbial lytic enzymes (Mehdy 1994, Hammond-Kosack and Jones 1996). H_2O_2, involved in cell wall fortification and hypersensitive response (HR) of barley attacked by the powdery mildew fungus, hampers fungal infection and, additionally, plays a role as signal for defense gene activation (Lamb and Dixon 1997, Hückelhoven and Kogel 1998, Hückelhoven *et al*. 1999, Grant and Loake 2000). Spore germination for a number of fungal pathogens has been shown to be inhibited by micromolar concentrations of H_2O_2 (Peng and Kuc 1992). In the apoplast, the accumulation of H_2O_2 may result by the activity of a plasma membrane NAD(P)H oxidases (Lamb and Dixon 1997), cell wall oxalate oxidases (Lane 1994), peroxidases (Bolwell *et al*. 1995), and FAD and copper-containing amine oxidases (Allan and Fluhr 1997, Laurenzi *et al*. 1999).

In the present research, using DDRT-PCR and RACE, we cloned the full-length cDNA of the novel gene from *Vitis pseudoreticulata* clone Baihe-35-1. As described in the sequence analysis mentioned above, GLOXrg is a

novel gene related to glyoxal oxidase. Conserved domain, glyoxaloxid N (pfam07250), was found in the deduced amino acid sequence. This single domain family represents the N-terminus (approximately 300 residues) of a number of plants and fungal GLOX. GLOX is an extracellular H_2O_2-producing enzyme found in lignino-lytic cultures of *Phanerochaete chrysosporium* as an essential component of its extracellular lignin degradation pathways (Kersten and Kirk 1987, Kersten 1990). Based on *GenBank* data, the researches on GLOX were focused on fungi. This is the first report on the cloning of the full-length GLOXrg cDNA from the plant except *Arabidopsis thaliana*, and only the deduced amino acid sequence of GLOXrg had 74 % identities to putative protein from *Arabidopsis thaliana* (similar to GLOX; accession No. CAB88357). Through mRNA differential display, GLOXrg was expressed more strongly in leaves inoculated with *Uncinula necator* for 1, 3, 5, 7, and 9 d than in control leaves of Baihe-35-1. The role of GLOXrg is probable to supply the H_2O_2 for the establishment of defense mechanism of grapevines. Given its limited lifetime and its toxicity potential, H_2O_2 must be generated in situ and the level of its accumulation must be finely regulated. GLOXrg probably plays a crucial role in the supply and the regulation of H_2O_2 levels in the extracellular matrix.

The GST gene fusion system is an integrated system for the expression, purification and detection of fusion proteins produced in bacterial, yeast, mammalian and insect cells (Sambrook *et al*. 1989, Ford *et al*. 1994, Jyrki and Hannu 2005). In this research, GLOXrg was cloned as fusion with GST, expressed, and analyzed by SDS-PAGE. About 83-kDa fusion protein was obtained and identical to the predicted size (predicted molecular mass is 57 kDa plus 26 kDa for GST), which revealed the correct expression of GST-GLOXrg fusion protein. Noticeably the yield of GST-GLOXrg fusion protein expressed in the GST gene fusion system was lower than the yield of GST, and we did not find the declining of bacteria's growth and reducing of plasmid copies during the culturing and inducing process. Probably the phenomenon is caused by the drop of tac promotor's transcription level and mRNA translation efficiency (Johnson *et al*. 1989). In order to improve the yield of GST-GLOXrg fusion protein, the optimal expression condition was optimized.

In this study, GST-GLOXrg fusion protein was expressed as insoluble inclusion bodies. The challenge was to convert the inactive and insoluble inclusion body protein aggregates into soluble, correctly folded biologically active products. Therefore, inclusion bodies were dissolved by high concentration of denaturing agent (guanidine hydrochloride) and then dialyzed against the concentration grades of guanidine hydrochloride. The dialysis condition was explored and improved.

In theory, GST-GLOXrg fusion protein can be cleaved by thrombin, but in fact the effect was not ideal in our study. Probably the phenomenon is because of the low cleavage efficiency and the digestion of protease. Immune response against GST label is weak per se (Lopez-Monteon *et al*. 2003), and immune response against GST-GLOXrg fusion protein treated with urea and DTT is predominantly directed to the GLOXrg moiety (Rothel *et al*. 1997), so purified GST-GLOXrg fusion protein treated with urea and DTT was concentrated by 12 % SDS-PAGE and then injected straight into New Zealand white rabbit as immunogen. The resulting polyclonal antibody has a high titer and the nicer specificity. It can be utilized for immunogold electron-microscopy localization of GLOXrg from *Vitis pseudoreticulata* clone Baihe-35-1 in the further studies. The availability of specific features of its spatial and temporal expression will further facilitate our ability to fully understand the exact physiological role of GLOXrg in mechanism of the resistance to the disease.

In conclusion, cloning, expression, purification and production of the polyclonal antibody of GLOXrg were accomplished. This is the first report on the cloning of the full-length GLOXrg from the plant except *Arabidopsis thaliana*, based on *GenBank* data. GLOXrg probably plays a crucial role in the resistance to *Uncinula necator* of Chinese wild *Vitis* species, through the differential expression. The following studies will be to utilize polyclonal anti-(GST-GLOXrg) for immunogold electron-microscopy localization and to obtain GLOXrg transgenic grapevine plants for detecting further its function of resistance to *Uncinula necator*. The researches of GLOXrg transgenic grape plants are being carried out in our laboratory. The availability of GLOXrg and preparation of the polyclonal antibodies of GLOXrg combined with other genes related to the resistance to *Uncinula necator* provide the excellent tools to the molecular breeding of highly disease-resistant grape varieties and the studies of the mechanism of the resistance to the disease of Chinese wild *Vitis* species.

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Basra, A.S. (ed.): **Handbook of Seed Science and Technology. -** Food Product Press, an Imprint of the Haworth Press, New York - London - Oxford 2006, 795 pp. USD 75.96. ISBN 13: 978-1-56022-314-6.

Remarkable progress has been made in the field of seed sciences in the last decades. Thus it is not surprising that a completely new handbook on seed science and technology was published only two years after the comprehensive "Handbook of Seed Physiology" (Benech-Arnold and Sánchez 2004). Both volumes were released by Food Product Press, which announces that the main focus of both new handbooks is on agriculture.

Indeed, the scope of the reviewed volume is to provide thorough seed-science background for applied research and practice. Nevertheless, impressively broad range of the topics addressed makes this book an invaluable source of information for basic research in seed biology. By dealing with ovule development, processes involved in seed germination and dormancy, and reviewing seed ecology and synthetic seed technologies this handbook covers all main topics of seed science. Despite such wide range of topics the handbook is quite well arranged, which is achieved by dividing according into four main sections.

Section I, "Seed developmental biology and biotechnology", focuses on different stages of seed development and associated cellular processes. The dynamic role of cytokinins or key role of carbon partitioning in developing seeds are shown at the cellular, biochemical and molecular levels. Special attention is paid to biotechnologies such as enhancement of seed nutritive value using genetic engineering or synthetic seed biotechnologies, documented by practical examples. In this respect Chapter 4, which explores models that can be used to determine grain numbers, fits less consistently to the general topic of the section and would be better placed in Section IV, which deals with classical technologies.

Section II, "Seed dormancy and germination", reflects recent molecular perspectives on the regulation and mechanisms of dormancy and germination, and explores the issue of hormonal signalling and interactions ruling these processes. Being myself an ecologist working on seed germination I found this part of the book most exciting. Another interesting chapter of this section addresses photoregulation of seed germination and explanation of the role of phytochromes in this process.

Section III, "Seed ecology", comprises different aspects of seed biology, such as competition for pollination and seed set, seed size, soil seed banks, seed predation or seed defence mechanisms. The topics are dealt with under a broad ecological context.

The last Section IV, "Seed technology", deals with special seed technologies used to assess seed quality and seed vigour, and conserve germplasm. Some techniques are commonly used, others result from recent technological development.

Individual chapters (25 contributions) are written as reviews of special topics with extensive reference lists which make the handbook a valuable overview of recent knowledge of seed biology and ecology. Moreover, many chapters end with future perspectives on the topic presented. The book has a strong applied facet (most information presented is discussed with practical implications for agricultural management), but it can be used as a source of information in all field of seed sciences. I can recommend it to undergraduate students, researchers and professionals working on various subjects of seed biology and ecology.

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