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Transcription of a β-1,3-glucanase gene in grape berries in a late developmental period, or earlier after wounding treatments

by

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S u m m a r y : The hydrolytic enzymes β-1,3-glucanases (EC 3.2.1.39) are known to be involved in plant defense reactions against pathogens and in developmental events. We have obtained two partial cDNA clones, corresponding to *Vitis vinifera* β-1,3-glucanase gene sequences (*VvGlu*1/7 and *VvGlu*26). The expression of *VvGlu*1/7 was studied in grape berries at different developmental stages and in wounded berries with or without salicylic acid elicitation. No constitutive expression was observed in young berries, whereas the induction of *VvGlu*1/7 transcription was detected during fruit ripening. By contrast, synthesis of mRNA coding for this isoform was generated in the first stage of rapid berry growth in response to wounding treatments with and without salicylic acid.

K e y w o r d s : berry, β-1,3-glucanase, berry development, defense, cell culture, DNA.

Introduction

The developmental processes of grape berries with regard to their defense mechanisms are of particular importance for the improvement of grape berry quality. However, limited data are available on the enzymes involved in defensive responses of grape berries during their development. In other plants studied to date, the expression of pathogen-related proteins (PRs) in response to pathogen's attack, wounding or elicitation, seems to be universal, and among them two important enzymes, chitinases and β-1,3-glucanases.

The involvement of β-1,3-glucanase in the active defense response of plants has been suggested to play a role against pathogens, either as a direct antimicrobial action in the breakdown of pathogen cell wall (KEEN and YOSHIKAWA 1983; MAUCH *et al.* 1988), or indirectly by releasing the elicitors from fungal cell walls (BOLLER 1987, 1988). β-1,3-glucanases were suggested to be also implicated in developmental processes, such as pollen development (BUCCIAGLIA and SMITH 1994), seed germination (VÖGELI-LANGE *et al.* 1994), and cell wall degradation during fruit softening (HINTON and PRESSEY 1980). The hormonal regulation of some β-1,3-glucanases by cytokinin and auxin was documented in cell cultures and in healthy plants (FELIX and MEINS 1986; VOGELSANG and BARZ 1993).

β-1,3-glucanase activity was found in a number of climacteric fruits, e.g. kiwi, (MCLEOD and POOLE 1994), peaches and tomatoes (HINTON and PRESSEY 1980), and in nonclimacteric fruits, e.g. grapefruit (MCCOLLUM *et al.* 1997). In the nonclimacteric grapevine, the expression of β-1,3-glu-canase was reported in leaves (RENAULT *et al.* 1996), embryogenic and nonembryogenic *in vitro* cell cultures (DELOIRE *et al.* 1997). We are interested in the β-1,3-glucanase expression in grapevine berries and in their possible role of the fruit's response to biotic and abiotic stresses.

Grape berries are characterized by three main periods of development (COOMBE 1976). Berry structure, metabolism and ability of tolerance to some phytopathogenic fungi differ in these periods (CHELLEMI and MAROIS 1992; KANELLIS and ROUBELAKIS-ANGELAKIS 1993; HARDIE *et al.* 1996). The aim of the present work was to determine the mRNA synthesis of grapevine glucanases in immature and mature berries, either elicited or not.

Here we report the molecular cloning and the sequence analysis of two partial cDNAs encoding β-1,3-glucanases in grapevine. We also present evidence for changes in the expression pattern of one β-1,3-glucanase mRNA in an early and a late stage of berry development, and after wounding of berries with or without salicylic acid treatment.

Material and methods

P l a n t c e l l c u l t u r e s : *In vitro* cell cultures, initiated from the skin of grape berries, *Vitis vinifera* cv. Gamay fréaux, were kindly provided by A. Latché (INRA, Toulouse, France). Multiplication subcultures were carried out according to AMBID *et al.* (1983), except that 80 ml of culture in 250 ml flasks were agitated at 120 rpm in an orbitary shaker. The cells were subcultured in one-week intervals. Seven-day-old cells were collected by vacuum filtration and used for RNA and protein extractions.

P l a n t m a t e r i a l a n d e l i c i t a t i o n : In 1995 and 1996 grape berries from *V. vinifera* L. cv. Chardonnay were harvested at the Agro.M vineyard (Montpellier, France). Three stages of fruit development were chosen according to the definition of LORENZ *et al.* (1995): "berry touch com-

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plete" (July); "beginning of ripening" (August); "berries ripe for harvest" (September).

Treatment was performed at the stage "berry touch complete" by salicylic acid (SA) injection with a syringe. At a similar stage of development samples of 100 berries from different bunches on 5 plants were treated as followed: 5 µl of 7.2 mM SA in potassium-phosphate buffer 15 mM, pH 6 were injected 2-3 times per berry. SA-treated berries were collected 7 d after treatment, frozen in liquid nitrogen and stored at -80 °C. In parallel, controls corresponding to healthy and wounded berries without SA were analyzed.

Total RNA extraction: For the extraction of RNA from Gamay freaux *in vitro* cell cultures, 1 g of dried cells was ground in liquid nitrogen and the powder added to 5 ml extraction buffer (200 mM Tris-HCl pH 8.5, 1.5 % (w/v) SDS, 300 mM LiCl, 10 mM Na_2EDTA, 1 % sodium deoxycholate, 1 mM ATA, 5 mM thiourea, 10 mM DTT and 1 % (v/v) NP-40) with 2 % (w/v) of PVPP. The aqueous phase was then submitted to several phenol-chloroform extractions. Total RNA was precipitated by LiCl at 2 M final concentration and resuspended in 1 ml of DEPC-treated water. RNAs were quantified by their absorbance at 260 nm. Seeds were removed from frozen berries before grinding. Total RNAs were extracted from berry tissues according to the protocol of TESNIÈRE and VAYDA (1991) with the modification of SARNI-MANCHADO *et al.* (1997).

Cloning of glucanase cDNAs by RT-PCR: Total RNAs extracted from *in vitro* cell culture were used as template for RT-PCR experiments. First strand cDNA synthesis was performed in a 20 µl final reaction volume, with 25 µg of total RNA as template, 750 ng of $oligo(dT)_{12-18}$ or 75 ng of random hexamers as primers and 200 U of Superscript II Reverse Transcriptase (GIBCO-BRL). Reaction conditions were as described by the manufacturer. Different dilutions of this cDNA reaction (1/10 or 1/50) were tested for the following amplifications. For PCR reactions, a set of degenerate primers were used designed as follows. The comparison of two conserved regions located at the 5' and 3' ends of the coding regions of plant β-1,3-glucanases (*Hevea brasiliensis* (U22147), *Nicotiana tabacum* (M59442), *Phaseolus vulgaris* (X53125) and *Nicotiana plumbaginifolia* (X54742)), allowed us to design degenerate primers for PCR reactions using the Infobiogen-Bisance programs package (France). The primer sequences were: gluc1: 5'-TA(T/C)AT(T/C/A)GC(T/C/A/G)GT(T/A)GG(T/C/A/G)AA(T/C)GA-3'; gluc3: 5' - CCA(T/C/A/G)CC(A/G)CT(T/C)TC(A/C/T/G)GA(A/T/C)AC(A/C)AC-3'. For PCR reactions, cDNA amplification was conducted in a 20 µl final reaction volume, containing 0.75 U of Taq polymerase (Promega), 10 pmol of each primer, 25 mM $MgCl_2$, 200 µM each deoxyribonucleotide triphosphate, overlaid with mineral oil, using a Mini cycler (MJ Research) apparatus. The PCR conditions were 95 °C for 5 min for denaturation, 48 °C for 1 min for primer annealing and 72 °C for 1.5 min for synthesis, for the first cycle and, 90 °C for 5 min, 48 °C for 1 min, 72 °C for 1.5 min, for the following 38 cycles. Primer extension was for 15 min for the last cycle. After electrophoresis of a part of the PCR reaction on 1 % agarose gel, amplified DNA was electrophoresed on 1 % NuSieve agarose gel (FMC Bioproduct, ME). The band of interest was excised from the gel and then purified using a GeneClean DNA purification system (Bio 101, La Jolla, CA). The purified fragment was ligated into the pGEM-T vector (Promega) and transformed into *E. coli* strain DH5α. After DNA extraction from positive clones the insert sizes were checked by restriction enzyme analysis. Twelve resulting clones were selected and analyzed. The clones were sequenced in both orientations using the dideoxychain termination method (Perkin-Elmer) with double-stranded DNA as a template, using either T7 or SP6 promoters as primers. Sequences were analyzed with the automated Applied Biosystem model 373A DNA sequencing system. Computer analysis was performed using the Infobiogen Network service (Paris, France), NCBI's BLAST (ALTSCHUL *et al.* 1990) and Clustal W 1.7 MSA (THOMPSON *et al.* 1994) programs.

Northern blot analysis: 10 µg of total RNA were loaded per lane and separated in denaturing 1.2 % agarose-formaldehyde gel, than transferred to a nylon membrane (Hybond-N, Amersham) and crosslinked by heating at 80 °C for 2 h. After 4 h prehybridization at 65 °C in a 5xSSC, 5xDenhardt's, 0.5 % SDS solution, containing 300 $\mu g \cdot m \cdot l^{-1}$ denatured salmon sperm DNA, filters were hybridized with ^{32}P-labeled DNA probes 16 to 20 h under the same conditions. Blots were washed successively at 65 °C for 2x20 min with 2xSSC, 0.1 % SDS, for 20 min with 1xSSC, 0.1 % SDS, and 5 min at room temperature with 0.1xSSC and exposed to X-ray film at -80 °C for at least 72 h and more.

Results

Cloning of partial cDNAs encoding grapevine β-1,3-glucanases: The RT-PCR strategy was successfully used to obtain grapevine glucanase cDNAs. Several independent RT-PCR reactions, with gluc1 and gluc3 primers, generated a band of the expected size of ca. 450 bp. After cloning, 12 transformants were analysed. Restriction mapping with *Sty*I, *Nco*I and *Not*I allowed us to distinguish between two cDNA groups. This was confirmed by sequence analysis. From each group, one of the cDNA clones was chosen and named respectively *VvGlu*1/7 (GenBank accession number U68144)) and *VvGlu*26 (GenBank accession number U73709). The clone *VvGlu*1/7 was obtained more frequently. These two cDNAs are different in length, with 441 bp for *VvGlu*1/7 and 468 bp for *VvGlu*26. The *VvGlu*1/7 clone presents 65.5 % identity to the nucleic acid sequence of the *VvGlu*26 clone, and 62.8 % identity to the corresponding deduced amino acid sequence. Alignment of these two cDNAs with sequences from the data base showed these clones to be related to plant β-1,3-glucanases. The identity percentages in nucleotide and amino acid sequences are presented in the Table. Comparison of the two grapevine glucanase sequences with homologous β-1,3-glucanases sequences from other plants available in the database revealed a nucleotide identity ranging from 55 to 71 % for *VvGlu*1/7 and from 58 to 72 % for *VvGlu*26. At the amino acid level the relative similarity was 49-65 % and 48-80 %, respectively.

*VvGlu*1/7 showed the highest degree of similarity with an homologous glucanase sequence from *Prunus persica*,

Table

Comparison of nucleic acid sequences and deduced amino acid sequences of *VvGlu*1/7 and *VvGlu*26 with the homologous sequences of genes coding for other plant β-1,3-glucanases

Plant	Nucleic acid identity (%)	Amino acid identity (%)	Accession number
*VvGlu*1/7			
Prunus persica	71.9	65.8	U49454
Nicotiana tabacum	68.3	63.0	X54456
Glycine max	68.1	64.7	U41323
Citrus sinensis	67.1	61.0	AJ000081
Lycopersicon esculentum	66.0	64.0	X74905
Hevea brasiliensis	65.0	62.0	U22147
*VvGlu*26			
Hevea brasiliensis	72.2	80.1	U22147
Phaseolus vulgaris	67.4	67.7	X53129
Pisum sativum	66.6	63.0	L02212
Arabidopsis thaliana	65.0	71.8	Z97340
Nicotiana plumbaginifolia	62.6	70.5	X54742
Solanum tuberosum	62.4	65.0	U01902

i.e. 71.9 % at the nucleotide level and 65.8 % at the amino acid level. *VvGlu*26 had 72.2 % identity with the *Hevea brasiliensis* glucanase nucleotide sequence and 80 % with the predicted amino sequence. The comparison of derived amino acid sequences for the grapevine glucanase cDNAs with the amino acid sequences of homologous plants glucanase domains is shown in Fig. 1.

The difference in length of the two partial cDNAs from grapevine, with 27 additional bases for *VvGlu*26, is derived from insertions of 9 residues at the deduced amino acid sequence. These insertions are observed in two regions. The first one, constituted of 4 residues, is located between position 13 and 14. At the same place, an insertion was also present in the *H. brasiliensis* glucanase, but was not found in other plant glucanases. The second region, with 5 additional residues between position 95 and 96 was present in all other plant glucanases chosen for comparison. We suggest that, for *VvGlu*1/7 residue deletion had occurred at position 95, whereas, for *VvGlu*26 residue insertion at position 13, could be at the origin of the differences observed between the clones.

Synthesis of β-1,3-glucanase mRNA in grape berries: Study of β-1,3-glucanase expression in berries was performed using *VvGlu*1/7 as a probe. Northern blots of total RNA extracted from grapevine berries showed a stage-dependent expression of the β-1,3-glucanase gene (Fig. 2). Under our conditions, no transcription was detected in green berries 4-5 weeks after anthesis. On the other hand, a tremendous hybridization signal was observed either at the beginning of ripening (8 weeks after anthesis) or in the ripening stage (about 15 weeks after anthesis). The size of the major band detected on gel blots was estimated to be ca. 1.4 kb. A second band at ca. 0.2 kb was observed in the berries at the end of ripening in September (Fig. 2).

By contrast to the absence of glucanase mRNA in green berries before ripening, wounding with or without SA resulted in a strong induction of β-1,3-glucanase transcription at this stage of development (Fig. 3). The synthesis of glucanase mRNA by wounding plus SA treatment was superior to wounding without SA.

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VvGlu1/7   YIPVGNEVSPSGA....QAQFVLPAMQNINNAISSAGLGNQIKVSTAIDTGVLGVSYPPSS     57
VvGlu26    --A-------VNGGTSRF--------R--RA-LA----QDRV-------LTL--N-----Q
H. brasil. --A----I--VNRGTAWL--------R--HD--R----QD---------LTLV-N-----A
G. max.    --S-----K-EHS....F---LV------QQ---N-------------E--A-AD-----M
P. pers.   --A-----K--DS....F---LV---R--QE---L---AKK-------------E-----I
N. tab.    --A-----K--DS....F---LV---R--QE---L---AKK-------------E-----I

VvGlu1/7   GSFKSGVL.SFLTSIISFLVKNNAPLLVNLYPYFSDLSN.....LNYALFTAPGVVVQDGQ    112
VvGlu26    -A-RGD-R.GY-DP--R---D-KS---A-I-----YSG-PKDIS-P------NS---W---
H. brasil. -A-RDD-R.-Y-DP--G--SSIRS---A-I----TYAY-PRDIS-P-----S-S---W---
G. max.    ---R-DYRTAY-DGV-RH--N--T-----V----AYINDPRNIS-D----RS-S------S
P. pers.   -----EYN.AL-YP--R---SHQS----------AYSG-TQDIR-D-------S------N
N. tab.    -----EYN.AL-YP--R---SHQS----------AYSG-TQDIR-D-------S------N

VvGlu1/7   LGYKNLFDAILDAVYSALERAGGSSLKIVVSESGW                            147
VvGlu26    R--Q-----M---L---------A--EV-I---
H. brasil. R--------T---L-------S-G--EV-------
G. max.    ---R-----MV----A---K---G-VS--------
P. pers.   F--R-----M--G--A---K---G---V-I--T--
N. tab.    F--R-----M--G--A---K---G---V-I--T--
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Fig. 1: Comparison of deduced amino acid sequences of two β-1,3-glucanases from grapevine (*VvGlu*1/7 and *VvGlu*26) with other plant β-1,3-glucanases: *Hevea brasiliensis* (U22146), *Glycine max* (U41323), *Prunus persica* (U49454), *Nicotiana tabacum* (M60402). Amino acids differing from *VvGlu*1/7 are shown. Identical amino acids are indicated by dashes (-). Numbers at the right side refer to amino acid positions in *VvGlu*1/7.

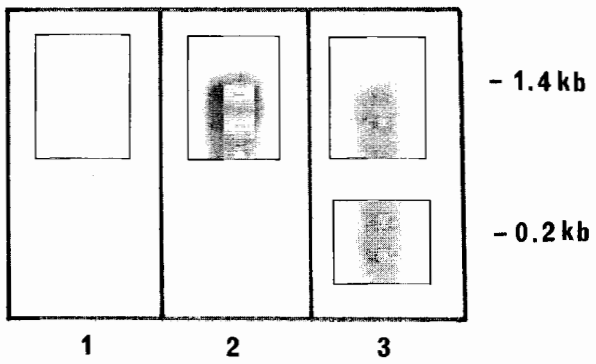

Fig. 2: Kinetics of *VvGlu* 1/7 β-1,3-glucanase mRNA synthesis during berry development: **1** - stage of "berry touch complete"; **2** - onset of ripening; **3** - stage of "berries ripe for harvest". Each lane was loaded with 10 µg of total RNA from berries without seeds. Blot corresponds to exposition of 4 d. RNA size (in kb) is indicated at the right side.

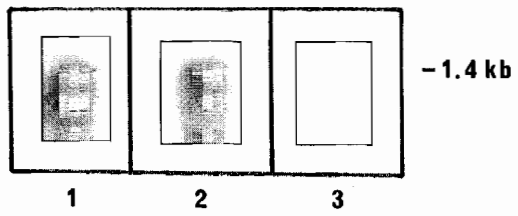

Fig. 3: Induction of *VvGlu*1/7 β-1,3-glucanase mRNA synthesis in young berries (stage of "berry touch complete") by elicitation and wounding. **1** - wounded berries; **2** - berries wounded and treated with salicylic acid; **3** - control berries. Each lane was loaded with 10 µg of total RNA from berries without seeds. Time after the treatment: 7 d.

Discussion

RT-PCR was successfully used to obtain partial cDNAs clones of β-1,3-glucanase from grapevine. Using one of the cDNAs as a probe allowed us to show some important features of the β-1,3-glucanase gene expression. Firstly, we have demonstrated that transcript levels were tremendously induced at the beginning of ripening, with a high expression level at berry maturity. Secondly, induction of synthesis of β-1,3-glucanases mRNA could be artificially obtained from green berries by wounding or wounding plus SA injection. These results questioned the role of β-1,3-glucanase during normal berry development or in response to elicitation.

There have been many reports on the identification and characterization of β-1,3-glucanases in plant-pathogen interactions or in developmental processes in vegetative parts of a number of plant species. Levels of β-1,3-glucanases are developmentally regulated in plant tissues and in cell cultures (FELIX and MEINS 1986; VOGELSANG and BARZ 1993; DONG and DUNSTAN 1997). However, only of a few papers reported the identification of PRs during berry development. Recently, TATTERSALL *et al.* (1997) have purified and identified *V. vinifera* thaumatin-like protein 1 (VVTL1) which was found in berries only. VVTL1 is a member of the PR-5 family of pathogenesis-related (PR) proteins and is expressed in a ripening-specific manner. ROBINSON *et al.* (1997) reported the cloning of two chitinase cDNAs from grape berries. Expression of these genes is induced at high levels throughout the sugar accumulation phase in grapes. The β-1,3-glucanase activity of berries was not detected in these experiments. On the other hand, different regulations of both, chitinase and glucanase activities have been reported in other nonclimacteric fruits such as grapefruit (*Citrus paradisi* cv. Marsh; MCCOLLUM *et al.* 1997). These authors have shown that the β-1,3-glucanase activity is lowest in young fruit and increases during fruit development. Also, at the late stage of grapefruit development, in crude flavedo extracts, a second band of protein was revealed by the immunodetection with glucanase antibodies.

In our case, preliminary experiments of β-1,3-glucanase immunodetection in the total soluble protein fraction of berries were performed with antiserum (provided by B. Fritig, CNRS, Strasbourg, France) raised against tobacco β-1,3-glucanases (data not shown). We have observed a protein band at 34 kDa reactive with anti-basic β-1,3-glucanase antiserum when the fruits were at "berries ripe for harvest" stage. We have not yet shown that this band corresponds to the expression of *VvGlu*1/7 mRNA glucanase, but recently RENAULT *et al.* (1997) have shown a correlation between glucanase mRNA expression, using the same probe, and the protein translation detected with the tobacco antibodies on grapevine leaves. The partial amino acid sequencing of this protein had 80 % of similarity with other plant β-1,3-glucanases.

It has been suggested that the enzyme endo-β-1,3-glucanase may participate in the dissolution of callose, a 1,3-β-glucosidically linked polymer of D-glucose units (KRABEL *et al.* 1993). However, the exact role of β-1,3-glucanases in plant development remains still unknown. More direct evidence for involvement of glucanases in protection against pathogenic fungi was demonstrated by their ability to hydrolyse glucans found in the fungal cell wall (MAUCH *et al.* 1988). It is also known that mechanical wounding in plants can induce rapid activation of genes that are involved in healing and in defense against a pathogen (GREEN and RYAN 1972; BOWLES 1991; BÖGRE *et al.* 1997). The induction of some PR proteins in plants was observed after exogenous salicylic acid treatments (for review: RASKIN 1992; JUNG *et al.* 1993; SIEGRIST *et al.* 1994). The accumulation of β-1,3-glucanases after SA elicitation or mechanical injury was reported in numerous plants (CABELLO *et al.* 1994; CRUS-ORTEGA *et al.* 1997; MÜNCH-GARTHOFF *et al.* 1997).

Our experiments have shown that β-1,3-glucanase gene expression was inducible by stress such as wounding with or without SA injection in berries during the first period of rapid berry growth. However, the level of β-1,3-glucanase mRNA synthesis was more important in case of SA elicitation.

In conclusion, our study supports the suggestion that from the onset of ripening (veraison) to maturity, there is a synthesis of PR-like proteins which are believed to play an antifungal and/or developmental role. Earlier induction of β-1,3-glucanase synthesis in immature berries due to wounding or chemical treatment, could be considered as a defensive response with a putative protection function.

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