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Identification and characterisation of a novel class I endo-β-1,3-glucanase regulated by salicylic acid, ethylene and fungal pathogens in strawberry

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Abstract. The identification of a full length cDNA encoding an endo-β-1,3-glucanase (*FaOGBG-5*) from strawberry (*Fragaria* × *ananassa* Duch) is reported. The analysis of the deduced amino acid sequence of FaOGBG-5 showed that it shares typical structural features and a high degree of identity with other plant β-1,3-glucanases of the class I. The expression of *FaOGBG-5* in plants infected with a virulent isolate of *Colletotrichum acutatum* and an avirulent isolate of *Colletotrichum fragariae* was examined. Induction of expression was observed with both pathogens but exhibited a delayed high expression with the virulent one. Additionally, the accumulation of *FaOGBG-5* transcripts was also observed after treatments with the stress related hormones salicylic acid and ethylene. Results obtained suggest that the β-1,3-glucanase encoded by *FaOGBG-5* may be implicated in plant defence against biotic and abiotic stress.

Additional keywords: plant defence, β-1,3-glucanases.

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Introduction

Plants are constantly challenged by a wide spectrum of pathogens including fungi, bacteria and viruses. The interactions of pathogens with plants can lead to compatible or incompatible reactions and whereas the former yields disease symptoms or the plant death, the latter brings about disease resistance. This scenario exposes that disease resistance in plants is a consequence of intricate signal transduction pathways controlled by linked resistance (*R* genes) and defence related genes (Jones and Dangl 2006). It has been demonstrated that a similar set of defence genes is activated in resistant and susceptible plants but with a different time-course and intensity (Jones and Dangl 2006). The defence response is, therefore, the result of an extensive genetic reprogramming, including *de novo* expression of many proteins, among which the pathogenesis-related (PR) proteins are included. PR proteins not only accumulate locally at the infection sites, but are also induced systemically throughout the plant (systemic acquired resistance, SAR) (Conrath 2006). Within this group of proteins there are many with catalytic activity such as the endo-β-1,3-glucanases (βGlu), also known as PR2 proteins, which are the most studied and have been reported to be required for resistance (Münch-Garthoff *et al*. 1997; Conrath 2006). Proteins of the family of β-1,3-glucanases (glucan endo-1,3-β-glucosidases, E.C. 3.2.1.39) catalyse the hydrolytic cleavage of β-1,3-D-glucosidic bonds of β-1,3-glucans; a major structural component of fungi cell walls (Leubner-Metzger and Meins 1999). Glucanohydrolases act in at least two different ways: directly by degrading the cell wall of pathogens; and indirectly by promoting the release of cell wall debris that can act as elicitors of defence reactions (Yamaguchi *et al*. 2000). Transgenic overexpression of β-1,3-glucanases in several plant species causes an enhanced resistance level (Leubner-Metzger and Meins 1999). After pathogen infection, many secondary signal molecules produced by plants can induce or repress βGlu expression, including jasmonic acid (JA; Wu and Bradford 2003) and salicilic acid (SA, Ward *et al*. 1991). There is also strong evidence suggesting that the expression and activity of βGlu proteins is increased in leaves of many plant species after a treatment with ethylene (Leubner-Metzger and Meins 1999).

Colletotrichum acutatum and *Colletotrichum fragariae* are two of the most important strawberry pathogen fungal species, as they cause anthracnose disease. In recent years, two β-1,3-glucanases of class II have been cloned and identified from strawberry (cv. Chandler). The expression of these enzymes is upregulated upon infection with both fungal pathogens, although

displays a higher induction with *C. fragariae* (Shi *et al*. 2006). Casado-Díaz *et al*. (2006) have also reported the cloning of an EST sequence corresponding to a putative β-1,3-glucanase that was downregulated upon infection with *C. acutatum* in cv. Camarosa.

The PCR-based approach using degenerate primers designed from the conserved regions of a certain gene family provides a convenient and efficient way of isolating homologues genes, especially in crops with complex genomes to which transposon tagging and map-based gene cloning strategies are not easy to implement. Many PR genes have been cloned, characterised and mapped using this strategy (Pflieger *et al*. 2001).

In our laboratory, using the degenerate primers approach, resistant gene analogues (RGAs) belonging to the nucleotide-binding site (NBS)-leucine-rich repeats (LRR) and serine/threonine protein kinases (STK) classes from wild and cultivated strawberries were isolated and characterised (Martínez Zamora *et al*. 2004, 2008). The aim of this work was to move one step forward in the investigation of strawberry defence genes by cloning PR2 sequences from cv. Pájaro. Results obtained let us to clone and identify a novel β-1,3-glucanase gene that is structurally different from other related genes reported in strawberry. A study of its regulatory pattern in response to infections with a virulent and an avirulent fungal pathogen and hormone treatments is also reported.

Materials and methods

Plant materials

Strawberry plants (*Fragaria* × *ananassa*) of the cv. Pájaro used in experiments were kindly provided by the strawberry BGA (Strawberry Active Germplasm Bank at University of Tucumán). Healthy plantlets were obtained from *in vitro* cultures in MS medium (Murashige and Skoog 1962), rooted in pots with sterilised substrate (humus: perlite, 2 : 1) and maintained at 28°C, 70% RH with a light cycle of 16 h per day. All senescent leaves were removed 10 days before the inoculation or hormone treatments, leaving only 3–4 young healthy leaves.

RNA extraction, degenerate PCR, cloning and sequencing

Total RNA was obtained and purified according to Iandolino *et al*. (2004) from strawberry young leaves from cv. Pájaro. RNA quantification was performed by spectrophotometry at 230, 260 and 280 nm. The degenerate PCR primers used to search for βGlu EST candidates were designed according to Pflieger *et al*. (2001), based on conserved amino acid sequence regions of 18 characterised β-1,3-glucanases. Primer sequences were PR2-S (5′-RYIGGWGTWTGYTAYGG-3′) and PR2-AS (5′-CADCCRCTYTCIGAYAC-3′). Reverse transcription reactions were conducted with 5 µg of DNase-treated total RNA, using SuperScript II RT (Invitrogen, Carlsbad, CA, USA) following recommendations of the manufacturer. PCR reactions were conducted in 50 µL total volume containing 200 ng of cDNA, 50 mM of KCl, 10 mM of Tris–HCl (pH 8.4), 2 mM of $MgCl_2$, 5 µM of each primer, 0.3 mM of each dNTPs and 2 U of Taq DNA Polymerase (Promega, Madison, WI, USA). PCR was performed in a PTC-100 thermal cycler (MJ Research Waltham, MA, USA). Cycling conditions included an initial denaturising step of 3 min at 95°C, followed by 35 cycles of: 45 s at 95°C (melting), 45 s at 45°C (annealing), 1 min at 72°C (extension) and a final extension step of 10 min at 72°C. After electrophoresis separation, a sharp band of the expected size (~700 bp) was excised from the gel and purified using the GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Sunnyvale, CA, USA). The purified DNA obtained for the band was cloned using the plasmid pCR2.1 of the TopoTA Cloning Kit (Invitrogen) following manufacturer recommendations and transformed into *Escherichia coli* DH5-α. Since the DNA obtained from the gel band may also contain different products of similar sizes, 40 recombinant bacterial colonies were isolated of further analysis. Recombinant plasmids were extracted using Wizard plus SV Minipreps DNA Purification System (Promega, Madison, WI, USA) and digested with *Eco*RI to verify the presence of the expected insert. Sequences of PCR products were determined using an ABI PRISM 377 DNA sequencer (Applied Biosystems, Foster City, CA, USA). To isolate the full-length cDNA of *FaOGBG-5*, RNA ligase-mediated rapid amplification of cDNA Ends (RLM-RACE) reactions were performed with the GeneRacer kit (Invitrogen) as recommended by the manufacturer, using 250 ng of DNAse (Ambion, Applied Biosystems) treated total RNA obtained from the cv. Pájaro. Based on nucleotide multiple sequence alignment, polymorphic regions were identified and a set of RACE primers specific for *FaOGBG-5* where designed (data not shown) following kit instructions; these primers were then used for 5′ or 3′ cDNA ends cloning.

Sequence edition and analysis

Clones were trimmed of vector sequence contamination using VecScreen at NCBI (http://www.ncbi.nlm.nih.gov/). Assemblage of DNA sequences and translation to the predicted amino acid sequence were performed using the DNAMAN software (ver. 6.0). Identity of the strawberry PR2 sequence obtained was confirmed by comparisons of DNA and amino acid sequences with sequences included in the GenBank NR database using BLASTN, BLASTX and BLASTP (Altschul *et al*. 1990) algorithms. Determination of conserved structural motifs in βGlu sequences was conducted with CDSearch (at NCBI, Marchler-Bauer and Bryant 2004). Multiple sequence alignments were performed with Clustal X (Thompson *et al*. 1997) and edited with BOXSHADE (http://www.ch.embnet.org/software/BOX_form.html). Construction of a neighbour-joining phylogenetic tree was conducted using the MEGA ver. 2.1 software (Kumar *et al*. 2001). Reliability of tree branches was evaluated using the bootstrap method (Felsenstein 1985) and Kimura's correction was applied. The SignalP server (Emanuelsson *et al*. 2007) was used to predict the presence and localisation of signal peptide cleavage sites. Potential *N*-glycosylation sites were identified by using NetNGlyc 1.0 (http://www.cbs.d.tu.dk/services/NetNGlyc). Nucleotide sequence of strawberry FaOGBG-5 was deposited in the GenBank Database under accession number JN204374.

Fungal cultures, inoculum preparation and plant inoculation

Two local fungal isolates characterised in our laboratory were used in this paper: the avirulent isolate M23 of *C. fragariae* and the virulent isolate M11 of *C. acutatum*. To prepare inoculum strains were propagated on potato dextrose agar (PDA) supplemented with streptomycin (300 μg mL^{-1}) and maintained at 28°C under continuous white fluorescent light (200 μmol m^{-2} s^{-1}) until the medium was completely covered. Then the culture surface was gently scraped with a sterile scalpel to remove conidia and suspended in sterile distilled water. The conidial suspension obtained was filtered through sterile gauze to remove mycelial debris under axenic conditions. The suspension was then diluted with sterile water (containing 0.01% Tween 20) to a final concentration of 1.5×10^6 conidia mL^{-1} and applied to plants as a spray to runoff (Salazar *et al*. 2007). Immediately after inoculation, plants were placed in a dew chamber at 100% RH, 28°C and a light cycle of 16 h per day (white fluorescent, 450 μmol m^{-2} s^{-1}) for 48 h. Plants were then moved to a growth chamber at 70% RH, 28°C and the same light cycle. Plants sprayed with sterile water containing 0.01% Tween 20 and incubated under same conditions were used as control. The susceptibility of plants towards pathogens was evaluated as a disease severity rating (DSR) according to the scale proposed by Delp and Milholland (1980).

Hormone treatments

Plants were sprayed to runoff with 5 mM SA in 30% methanol and maintained for 72 h in a growth chamber as mentioned above. Plants treated with 30% methanol and incubated under the same conditions were used as control. For ethylene treatment experiments, plants were placed in a sealed chamber along with a small beaker containing 10 mL of 0.1 M Ethephon solution (an ethylene gas-releasing reagent) or with 10 mL of 0.15 mM 1-MCP (1-methylcyclopropene, a gaseous competitive inhibitor of ethylene action), let plant leaves get in contact with the gaseous hormones for 24 h and then moved to a growth chamber as mentioned above. Control plants were incubated under the same conditions without Ethephon or 1-MCP. After SA, Ethephon or 1-MCP treatments, a group of plants was inoculated with the virulent isolate M11 of *C. acutatum* (as described above) for DSR estimation and another group was used for RNA extraction and expression analysis.

Expression analysis of FaOGBG-5 *in leaves*

Total RNA was obtained from strawberry leaves at 24, 48, 72 and 96 h after inoculating with the isolates M23 or M11 as described above and also after treating with SA, Ethephon, 1-MCP and their controls. The semiquantitative RT–PCR method was used to evaluate the expression of *FaOGBG-5* gene and compared with the expression pattern of the gene *FaBG2–3* previously characterised by Shi *et al*. (2006). *GAPDH1* gene (AF421492) was used as the charge control of each treatment and to attain the same PCR exponential phase. Specific primers used were: GAPDH1F: 5′-CTACAGCAACACAGAAAACAG-3′, GAPDH1R: 5′-AACTAAGTGCTAATCCAGCC-3′, FaOGBG-5F: 5′-CCTTCAAAGAACCACC-3′, FaOGBG-5R: 5′-CAC ATCTCTGGCACAG-3′, FaBG2–3F: 5′-CTCCATTGTTGCC CAA-3′ and FaBG2–3R: 5′-AACCCTACTCGGCTGA-3′. The PCR program used was: 7 min at 94°C (initial denaturing step); 32 or 30 cycles (32 for *FaOGBG-5* and 30 for *FaBG2–3* and *GAPDH1*) of 45 s at 94°C (melting), 1 min at the corresponding annealing temperature (see below) and 1 min at 72°C (extension) and a final extension of 10 min at 72°C. The number of cycles used for each gene was adjusted to obtain the specific band amplified at the exponential phase of PCR reaction. Annealing temperatures for *FaOGBG-5*, *FaBG2–3* and *GAPDH1* genes were 52, 53 and 52°C respectively. Amplified bands were visualised by agarose gels (2%) ethidium bromide-stained (10 μg mL^{-1}) and photographed under UV light with a digital camera. Bands observed were digitalised and quantified using Total Laboratory Quant software (Nonlinear Dynamics Ltd, Newcastle, UK). Relative expression of studied genes was estimated as the ratio of gene expression change between inoculated/treated and not inoculated/untreated (control) plants with respect to their control. To ensure the absence of genomic DNA in each cDNA sample, *GAPDH1* primer sequences were designed to enclose an intronic region.

Measurement of SA

Content of SA in inoculated plants with M23 or M11 isolates was measured at 0, 24, 48 and 72 h post inoculation (HPI). Plants treated with SDW plus 0.01% Tween 20 subjected to the same experimental conditions were used as not infected control plants. The SA amount was determined from phloematic exudates following the procedures recommended by Richardson *et al*. (1982), modified in our laboratory. Petioles of strawberry leaves were excised at 1 cm from the plant crown and phloematic exudate from basal tip was collected using a micropipette. Collected exudates were immediately dispensed into 1 mL of ice-cold ethanol absolute, acidified with HCl to pH 3.0. Extracts were pooled and the precipitate eliminated by centrifugation at 10 000*g* for 15 min. The supernatant was transferred to a new tube, completely dried under vacuum and the DW of each sample was determined. Sample were then dissolved in 1 mL of 30% methanol and used for HPLC analysis. SA was separated by high performance liquid chromatography using a C18 reversed phase column (Prodigy 5 ODS-2, Phenomenex, Torrance, CA, USA), with a gradient of water with 0.1% TFA (trifluoroacetic acid, solvent A) and methanol with 0.1% TFA (solvent B). The gradient was performed from 30% to 100% of B in 20 min and then maintained for 10 min. SA was detected at 302 nm and collected fractions analysed by fluorometry (λex = 296 nm; λem = 408 nm) for quantitative determination, using a spectrofluorometer (Photon Counting PC1, ISS, Owingen, Germany). SA content in each sample was estimated by using standard SA solutions subject to HPLC and fluorometric analysis. The latter procedure allowed us to estimate the losses during the manipulation of samples. Results were referred as ng of SA per mg of exudate DW.

Statistical analyses

Phytopathogenic tests were randomised with eight plants per pathogen or treatment evaluated and per experimental unit; four plants were used for the inoculation/treatment and four

as control plants (see above). Experimental data obtained were analysed with the program Statistix (Analytical Software 1996); a least significant difference (l.s.d.) test was used for determining the arithmetic mean of the DSR value (significance level, 0.05) of plants inoculated with each isolate and an ANOVA was used for evaluating the data dispersion with respect to the mean value. DSR and relative expression estimates correspond to average values obtained from three independent experiments.

Results

Isolation of β-1,3-glucanase gene analogues from strawberry

Amplification of strawberry cv. Pájaro cDNA with the degenerate primers yielded a unique DNA fragment of ~700 bp. After cloning, the characterisation of the amplicon revealed that each band contained many DNA fragments of the same size. Of the 40 putative strawberry βGlu clones sequenced, 37 had significant BLASTN hits in the GenBank NR Database with β-1,3-glucanases genes or related sequences from other species such as *Populus* spp., *Vitis* spp. and *Arabidopsis* and with high identity levels (data not shown). Further analysis showed that from the 37 sequences mentioned above 11 had potential stop codons or frame shift mutations, whereas the remaining 26 presented uninterrupted open reading frames. Nucleotide sequence identity among these sequences ranged between 59 and 100%. Clones with amino acid identities above 98% were considered identical; therefore, only five sequences were included in the analysis (*FaOGBG-1* to *-5*). When compared with protein databases by BLASTX, the deduced amino acid sequences of clone FaOGBG-4 presented 98.89% identity with β-1,3-glucanase ToyoGluIII (BAC66184) whereas FaOGBG-1, FaOGBG-2 and FaOGBG-3 exhibited high identity (>99%) with the glucanases of the class II: FaBG2–1 (AAO16642), FaBG2–2 (AAX81589) and FaBG2–3 (AAX81590) reported by Shi *et al*. (2006). Only the clone FaOGBG-5 exhibited a low identity scores with previously cloned sequences, showing a maximum identity score of 53% with respect to FaBG2–3. Furthermore, when the clone FaOGBG-5 was compared with ESTs included in other databases, results indicated a high identity (97%) with a cold-stressed seedling EST of the related species *Fragaria vesca* (DY671255).

Cloning and structure analysis of FaOGBG-5

For isolating the full-length cDNA of FaOGBG-5, 5′ and 3′ RLM-RACE was performed with the GeneRacer kit (Invitrogen). The obtained sequence consisted in a 1259 bp fragment (GenBank accession number JN204374), presenting a 5′ UTR (untranslated region) of 30 bp, a 1116 bp coding region and a 3′ UTR of 87 bp, preceding a poly(A) tail of 26 a residues (data not shown). The sequence of FaOGBG-5 encodes a putative signal peptide of 33 amino acids as determined by SignalP and the mature protein contains 338 residues (Fig. 1) with a predicted molecular weight of 36.96 kDa and an estimated isoelectric point (pI) of 6.65. CDSearch identified between the residues 35 and 314, with a highly supported value (Evalue: 1.12 e-129), the characteristic glyco-hydro-17 domain typical to the glycosyl-hydrolases superfamily 17 (pfam: 00332).

A multiple sequence aligment with β-1,3-glucanases from class I (Gn1 of *Nicotiana plumbaginifolia* (AAA34078) and Gln2 of *Nicotiana tabacum* (X53600)), class II (AAR06588 from *Vitis riparia* and FaBG2–3 (AAX81590) from *F. ananassa*), class III (tobacco PR-Q' (CAA38324) and tomato PR-Q'b (CAA52871)) and class IV (tobacco sp41a (CAA38302) and Tag-1 (CAA82271)) allowed us to identify within the sequence of FaOGBG-5 the *C*-terminal extension typical of the class I β-1,3-glucanases (Fig. 1). Additionally, the NetNGlyc 1.0 server predicted a single potential *N*-glycosylation site in the context Asn-X-Ser/Thr (where 'X' corresponds to any amino acid except Pro) at the position N362 (Fig. 1) at the *C*-terminal extension. Finally, by comparing the amino acid sequence of FaOGBG-5 with the sequence of barley β-1,3-glucanase GII (P15537), whose crystal structure was resolved by Varghese *et al*. (1994), two essential conserved glutamic residues at the active site, E273 and E330 and two other important catalytic residues at E322 and K325 were identified (Fig. 1). In Fig. 2 we present the neighbour joining phylogenetic tree based on the amino acid alignment showed in Fig. 1. Figure 2 shows that the sequences are grouped in four clusters according to their class, with the newly identified β Glu (FaOGBG5) included in the same group of Gn1 and Gln2, two glucanases of the class I identified in *N. plumbaginifolia* and *N. tabacum* respectively. Based on all the information obtained we concluded therefore, that the sequence identified as FaOGBG-5 corresponded to a β-1,3-glucanase of the class I.

Expression analysis of FaOGBG-5

Since the expression of *FaBG2–3* was already studied by Shi *et al*. (2006) in strawberry plants of the susceptible cv. Chandler infected with virulent isolates of *C. fragariae* and *C. acutatum* and we also detected the expression of this gene in the cv. Pájaro, the expression of the newly found gene *FaOGBG-5* (Fig. 3*a*) was studied in comparison with *FaBG2–3* (Fig. 3*b*). Accordingly, semiquantitative RT–PCR assay was used to study the time-course expression of both genes in the cv. Pájaro at 0, 24, 48 and 72 h after challenging with the avirulent isolate M23 of *C. fragariae* or the virulent isolate M11 of *C. acutatum*. Results showed that the expression of *FaOGBG-5* exhibited a maximum at 48 HPI in strawberry plants inoculated with M23, whereas plants inoculated with M11 exhibited a very strong induction at 72 HPI (Fig. 3*a*). Noteworthy was that the latter coincided with the appearance of the first disease symptoms as shown in the insert of Fig. 5. In comparison, the expression of *FaBG2–3* showed a complete different profile. *FaBG2–3* was almost not affected by the infection with both pathogens, exhibiting a very weak upregulation at 48 HPI when plants were infected with M23 (Fig. 3*b*).

Since the isolates M23 and M11 are hemibiotrophic pathogens causing incompatible or compatible interaction, respectively, we were interested to investigate whether different defence signalling pathways may be implicated (Singh *et al*. 2011). Accordingly, experiments were conducted to evaluate the effect of salicylic acid, ethylene and the ethylene antagonist 1-MCP on the expression of *FaOGBG-5* and *FaBG2–3*. Results revealed that each hormone influenced differently the expression of *FaOGBG-5* and *FaBG2–3* (Fig. 4). *FaOGBG-5* was highly and moderately

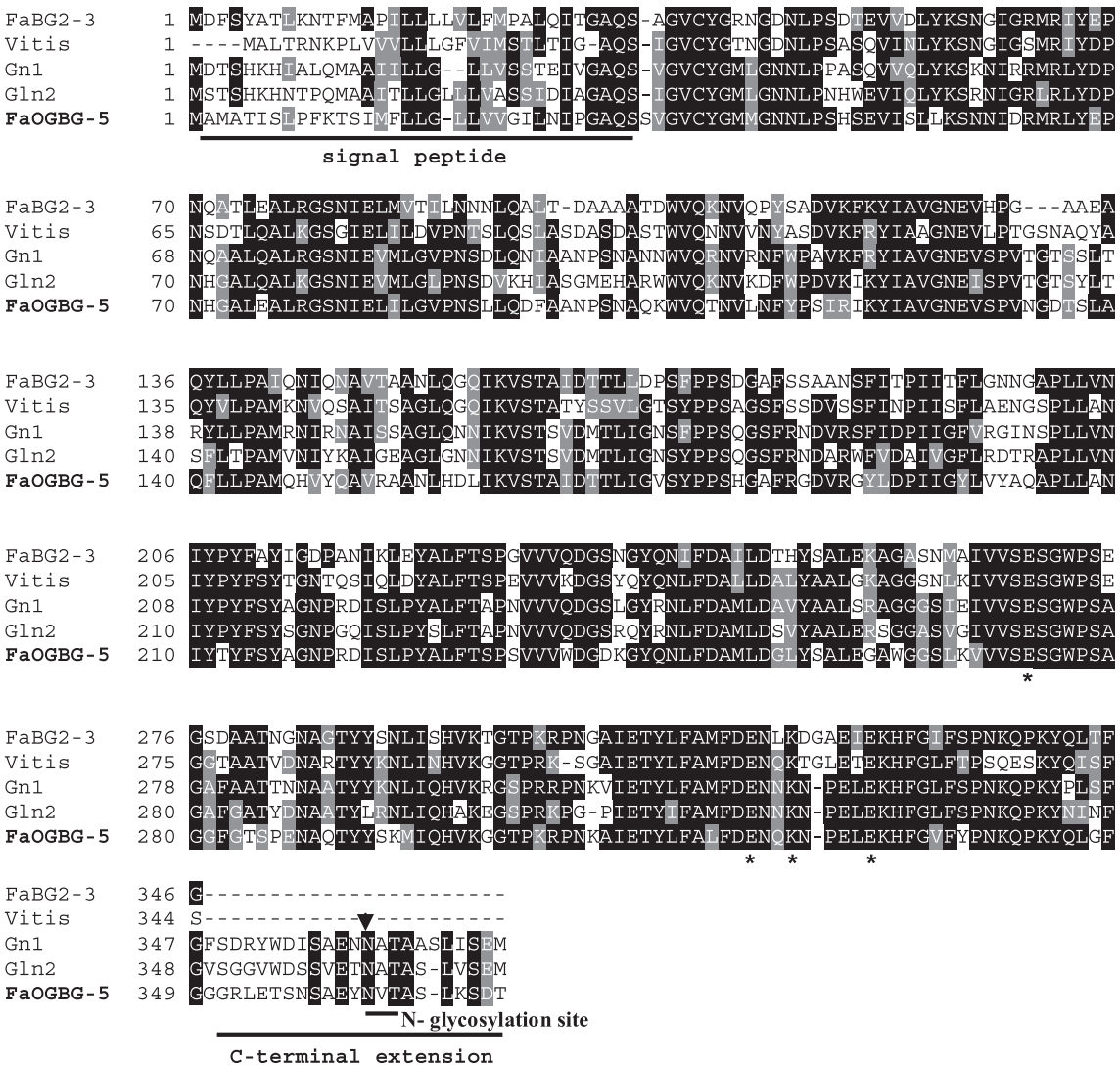

Fig. 1. Alignment of the deduced amino acid sequence of FaOGBG-5 obtained from strawberry cv. Pájaro with sequences of β-1,3-glucanases of class I: *Nicotiana plumbaginifolia* Gn1 (AAA34078) and *N. tabacum* Gln2 (X53600), class II: *Vitis riparia* (AAR06588) and *F. ananassa* FaBG2–3 (AAX81590), class III: tobacco PR-Q' (CAA38324) and tomato PR-Q'b (CAA52871) and class IV: tobacco sp41a (CAA38302) and Tag-1 (CAA82271). Fully conserved residues are shaded in black and semi-conservative substitutions are shaded in grey. The conserved active site and essential catalytic residues (E or K) are indicated by asterisks. Signal peptides and *C*-terminal extensions of class I representatives are underlined. Within *C*-terminal extension the predicted and conserved N-linked glycosylation sites are underlined and the arrow indicates the asparagines candidate to be glycosylated.

expressed after treatment with SA and Et, respectively (Fig. 4*a*), however, expression of *FaBG2–3* was unchanged (Fig. 4*b*). With the aim to confirm the participation of ethylene on the induction of *FaOGBG-5* the ethylene receptor inhibitor 1-MCP was used. Figure 4*a* shows that whereas 1-MCP produced a slight downregulation of *FaOGBG-5*, it had no effect on the expression of *FaBG2–3* (Fig. 4*b*).

Effect of SA and Et on the susceptibility of the cv. Pájaro to the virulent isolate M11

Since results shown in Figs 3 and 4 suggested that the expression of *FaOGBG-5* may depend on the activation of both defence signalling cascades (e.g. SA and Et) we tested whether plants exogenously treated with SA or Et could acquire resistance to the isolate M11. Figure 5*d* shows that plants treated with SA acquired a higher resistance (DSR = 2) to M11 whereas plants treated with Et or 1-MCP did not. This result clearly shows that the defence response observed is activated by SA, endow plants with an effective mechanism to repel M11 attack and the latter is associated with the expression of *FaOGBG-5*. By contrary, plants treated with Et (Fig. 5*e*) or 1-MCP (Fig. 5*f*) cannot halt the attack of M11 (at least under the experimental conditions used). In Fig. 5 we also present the results obtained when plants of the cv. Pájaro were treated only with water (Fig. 5*a*) or M23 (Fig. 5*b*) as control experiments. In the latter case, plants

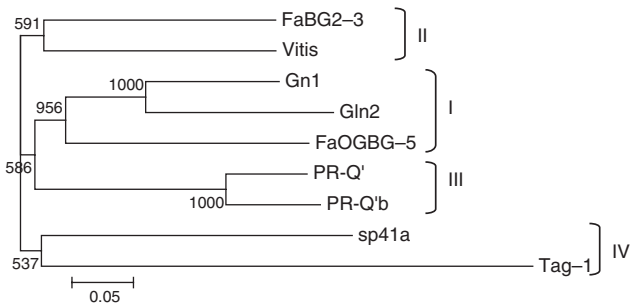

Fig. 2. Phylogenetic tree of β-1,3-glucanases classes I–IV obtained by the neighbour-joining method. Numbers on the branches indicate bootstrap values (1000 iterations). Accession numbers of the β-1,3-glucanases used correspond to those used in Fig. 1 (see 'Materials and methods').

did not show symptoms of the disease, indicating that the M23 behaves as an avirulent pathogen and cannot undergo into the necrotrophic phase as M11 did (Fig. 5*c*). In the insert of Fig. 5, the evolution of the disease symptoms during the first 15 days after M11 infection is displayed and as we mentioned earlier the very first symptoms of the disease were detected 3 days after the infection.

Accumulation of SA in M23 treated plants

Experiments conducted to estimate the level of SA in the phloematic fluid of plants of the cv. Pájaro infected with M23 or M11 showed that whereas the former brought about the increase of SA with a clear maximum at 48 HPI the latter did not (Fig. 6). This outcome led us to conclude, therefore, that the interaction with M23 causes the activation of a SA dependent defence response in this cultivar, strongly supporting the results presented in Figs 4 and 5. In contrast, plants exposed to M11 failed to accumulate SA (Fig. 6) exhibiting a susceptible phenotype.

Discussion

In this paper we report the identification and characterisation of a novel class I β-1,3-glucanase cDNA (*FaOGBG-5*) from strawberry using the combination of degenerate primers PR2-S and PR2-AS, designed to anneal conserved motifs found in endo-β-1,3-glucanase genes of different species.

The cDNA sequence has an ORF encoding a protein of 371 amino acid residues preceded by a signal peptide of 33 residues (Fig. 1). The predicted molecular weight and pI of the mature FaOGBG-5 protein of 338 amino acid residue are 36.96 kDa and 6.65 respectively. The identification and conservation of the two essential glutamic residues (E273 and E330) that have been suggested to conform the polysaccharide substrate binding site and two other important catalytic residues (E322 and K325) suggests that FaOGBG-5 is a functional enzyme. The endo-β-1,3-glucanase genes identified so far encode mature proteins that were grouped in four classes according to their size, structure, pI, cellular localisation and regulatory differences (Leubner-Metzger and Meins 1999). Class I, β-1,3-glucanases (βGluI), are mostly basic vacuole-localised proteins that have been shown to inhibit the growth of certain pathogenic fungi *in vitro* (Leubner-Metzger and Meins 1999). In contrast, most class II, III and IV β-1,3-glucanases are acidic extracellular proteins that have no inhibitory activity on fungal growth in *in vitro* assays (Leubner-Metzger and Meins 1999). Additionally, class I β-1,3-glucanases present a characteristic N-glycosylated *C*-terminal extension with a signal for vacuolar transport which is absent in the other classes (Leubner-Metzger and Meins 1999). Although FaOGBG-5 is not a basic βGlu, based on the predicted pI of the mature protein and the activity has not been proved yet, its structure, the presence of the *C*-terminal extension and the conserved position of a putative N-glycosylation site (see Fig. 1) indicate that this enzyme is most likely a member of the class I β-1,3-glucanases and the first reported in strawberry.

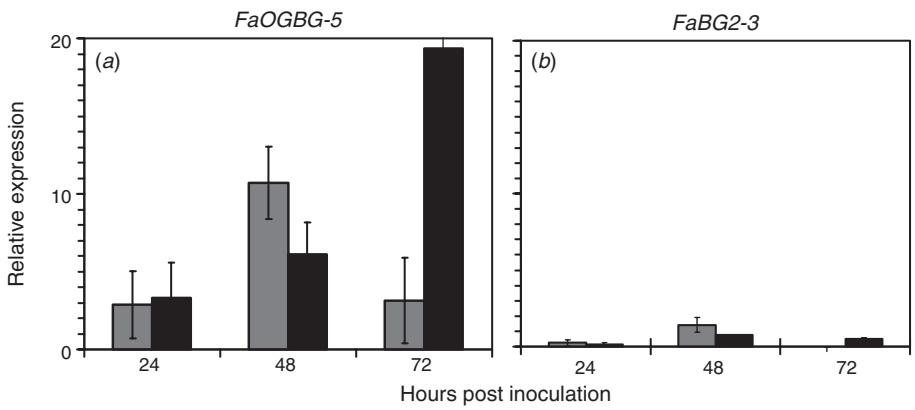

Fig. 3. Time course expression of *FaOGBG-5* and *FaBG2–3* genes in response to fungal inoculations of strawberry plants of the cv. Pájaro with *C. fragariae* M23 and *C. acutatum* M11. Expression of *FaOGBG-5* and *FaBG2–3* was evaluated in leaves 24, 48 and 72 h after M23 (grey bars) or M11 (black bars) inoculation. Relative expression was calculated as the ratio of gene expression change in inoculated/treated plant with respect to their control. Results correspond to average values coming from four replicates and two independent experiments ($n = 6$).

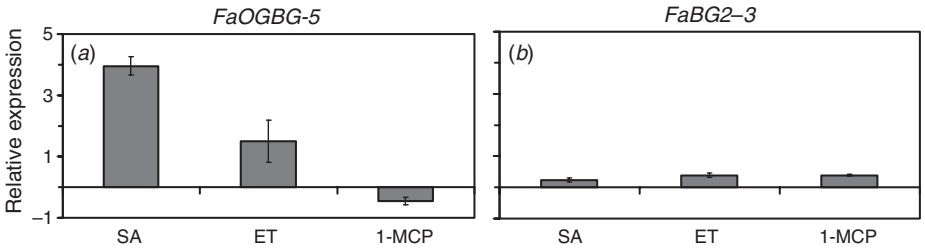

Fig. 4. Expression level of *FaOGBG-5* and *FaBG2–3* genes in response to hormone treatments. The expression was analysed in strawberry plants of the cv. Pájaro 72 h post-treatment with salicylic acid (SA) and 24 h post treatment with ethylene (Et) and 1-methyl cyclopropene (1-MCP). Relative expression was calculated as the ratio of gene expression change in inoculated/treated plant with respect to their control. Results correspond to average values coming from four replicates and two independent experiments ($n = 6$).

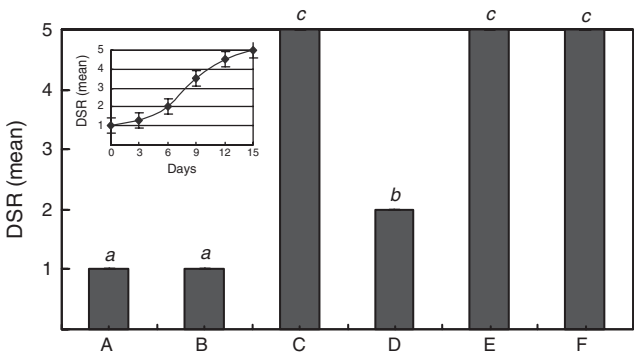

Fig. 5. Disease severity rating (DSR) of the cv. Pájaro of strawberry when treated with (**A**) water, (**B**) the isolate M23 of *C. fragariae*, (**C**) the isolate M11 of *C. acutatum* and inoculated with M11 seven days after treatments with (**D**) SA, (**E**) ethylene and (**F**) 1-MCP. DSR values were evaluated 50 days after treatments. DSR values with different letters are statistically different (Tukey's test, $P = 0.05$). The insert depicts the time-course evolution of disease symptoms caused by the virulent isolate M11 evaluated as DSR.

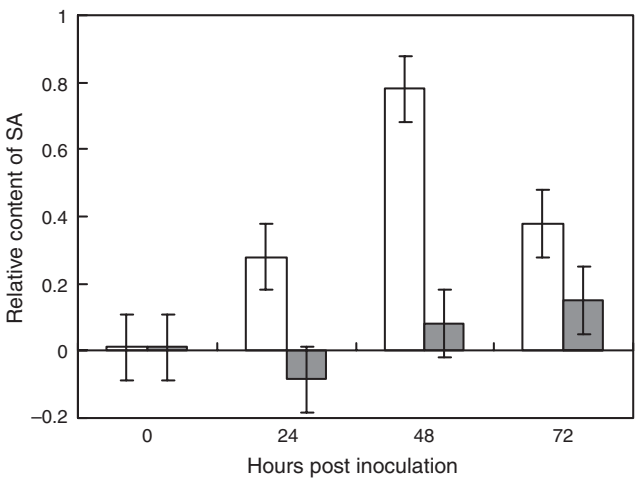

Fig. 6. Time course change of SA content in floematic exudates of strawberry plants (cv. Pájaro) inoculated with M23 (white bars) or M11 (grey bars) fungal pathogens. Results are expressed as the variation of SA content in inoculated with respect to control plants. Values represent the average of three experimental replicates ($n = 6$) with standard deviation $\sigma_{n-1} = 0.2$.

It has been reported that the expression of β-1,3-glucanases depends on various factors, such as plant species and cultivar, pathogen isolate, the particular βGlu class and other stimuli such as hormone treatments or abiotic stress (Philip *et al*. 2001). It has also been shown that β-1,3-glucanases are constitutively expressed at low levels, but are strongly induced upon pathogen infections (Ward *et al*. 1991). Consistent with this information, an increase of expression of *FaOGBG-5* was observed after the infection with both *Colletotrichum* species, although with different patterns. Whereas the avirulent isolate M23 provoked a maximum expression at 48 HPI, the virulent M11 caused a strong upregulation at 72 HPI. Although in a first glance the expression pattern observed for *FaOGBG-5* until 48 HPI would agree with results by Shi *et al*. (2006), for *FaBG2–3* we observed that after 48 HPI plants infected with *C. fragariae* (M23) exhibited a downregulation of both genes whereas in plants inoculated with *C. acutatum* (M11) the expression of *FaOGBG-5* experiments a strong upregulation that was not observed for *FaBG2–3*. This outcome appears to be contradictory if we assume that FaOGBG-5 was a protein involved in plant defence; the delayed detected in induction of *FaOGBG-5* would allow M11 to grow and the disease progress. A plausible explanation is found if we consider the strategies deployed by plants to resist the attack of hemibiotrophic pathogens such as *Colletotrichum* fungi (Kliebenstein and Rowe 2008). However, further investigation would be required to elucidate this behaviour.

From results shown in Figs 4, 5 and 6 we concluded that the expression of *FaOGBG-5* relies mainly on SA dependent response and since M23 exhibited a biotrophic way of life, the activation of SA dependent defence response would prevent its growth, turning the latter an avirulent one (Fig. 5). In contrast, results shown in Fig. 3*a* suggest that M11 somehow can delay the induction of *FaOGBG-5* by suppressing temporarily the SA dependent response. This situation would enable M11 to accomplish the first biotrophic phase, allowing the pathogen to advance on the necrotrophic phase. This speculation is supported by the results presented in Fig. 5*d*, where an increase of the resistance against M11 in plants exogenously treated with SA was observed.

We hypothesise, therefore, that this effect can be attributed to the action of a virulence factor produced by M11 that would cause the transient suspension of the SA dependent defence response, preventing thereby the prompt expression of

FaOGBG-5 in response to the pathogen attack (as displayed by M23, Fig. 3*a*), releasing the control when the pathogen undergoes into the necrotrophic phase. This scenario let us further speculate that the expression of *FaOGBG-5* may not only be under the control of such hypothetical defence inhibitory factor, but also under the control of an elicitor produced by the pathogen or the plant after the first symptoms of necrosis of plant tissues as reported for 'defence secondary elicitors' (Ron and Avni 2004).

Experiments conducted in our laboratory and previously reported by Chalfoun *et al*. (2005) and Salazar *et al*. (2007) bring some support to the first hypothesis. These authors showed that the virulent isolate M11 of *C. acutatum* prevented the onset of the defence response induced by the avirulent isolates F7 and M23 when inoculated simultaneously. In contrast, the fact that the isolate M23 does not induce a strong upregulation of *FaOGBG-5* and slightly decreases at 72 HPI may indicate that, since the avirulent isolate cannot undergo into a necrotrophic phase the hypothetical fungal/plant secondary elicitor would not be produced. Noteworthy is the correlation observed between the strong induction of *FaOGBG-5* at 72 HPI with the appearance of the first disease symptoms provoked by M11 (see insert of Fig. 5), in contrast to the behaviour observed with M23. This fact provides some support to the second hypothesis about the participation of a secondary defence elicitor.

Taken together, with these considerations and the results obtained we are tempted to speculate that although evidence indicate that SA is an important regulator of *FaOGBG-5*, a secondary elicitor participates on its expression. Questions arise, however, about the real role of FaOGBG-5 in defence, as it is expressed very late when the pathogen has already entered into the necrotrophic phase affecting the plant and no signal of recovery was observed despite the high induction of *FaOGBG-5*. More experiments are required to clearly establish which of these two hypotheses explain the results obtained, although we cannot rule out that both may take place at different times.

In the case of *FaBG2–3*, the repression after infection with the avirulent *C. fragariae* (M23, Fig. 3*b*) agree with the results reported by Casado-Díaz *et al*. (2006) who identified a putative β-1,3-glucanase EST which is downregulated upon infection with *C. acutatum* in the susceptible cultivar Camarosa. Ward *et al*. (1991) reported that tobacco β-1,3-glucanase and chitinase of the class I are weakly or not induced by SA. In our case, we have observed a rather different behaviour for the *FaOGBG-5* gene which is strongly induced by SA (Fig. 4*a*). Treatments with ethylene have been shown to elevate the mRNA levels of βGluI, but not of βGluII or βGluIII in leaves of many species (Leubner-Metzger and Meins 1999). In agreement with those observations, our experiments revealed that whereas the class I *FaOGBG-5* is moderately induced by Et (Fig. 4*a*), the previously reported class II *FaBG2–3* (Shi *et al*. 2006) was not induced in the cv. Pájaro. The latter was confirmed with the results obtained: expression of both genes in the presence of 1-MCP. Results showed that 1-MCP reversed the effect produced by the Et in *FaOGBG-5*, as expected for an inhibitor of the ethylene receptor, whereas the gene *FaBG2–3* did not show any significant effect.

As mentioned above, the nucleotide sequence of *FaOGBG-5* presented 97% identity with an EST of the related species *Fragaria vesca* obtained from cold-stressed plants cDNA library (Casado-Díaz *et al*. 2006). Using the EST sequence (DY671255) and the recently sequenced *F. vesca* genome a BlastN analysis was conducted. The outcome let us identify the corresponding genomic sequence, which presented an overall 96% identity with *FaOGBG-5*, suggesting that it may correspond to a orthologue gene. In the upstream region of the genomic sequence of *F. vesca* orthologue various *cis*-acting regulatory elements were identified (data not shown). Two GCC-boxes corresponding to ethylene/jasmonate responsive element found in many pathogen-responsive genes (Brown *et al*. 2003) and two W-box specifically recognised by salicylic acid (SA)-induced WRKY DNA binding proteins (Rushton *et al*. 1996) were detected. Although some sequence difference between *F. vesca* and *F. ananassa FaOGBG-5* orthologs should be expected, the phylogenetic proximity of these species and the lack of more information of *F. ananassa*, makes reasonable to use *F. vesca* as a source of sequence information. Hence, these results provided further support about the effect of SA and ET on the expression of *FaOGBG-5* in the cv. Pájaro of *F. ananassa*.

In conclusion, a cDNA encoding for a novel strawberry endo-1,3-β-glucanase (*FaOGBG-5*) exhibiting typical features of the class I is presented. Our results highlight the main structural and regulatory difference observed between the class I FaOGBG-5 and class II FaBG2–3 glucanases studied, suggesting functional differences as defence proteins.

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