Functional characterization of a defense-related *class-III chitinase* promoter from *Lupinus albus*, active in legume and monocot tissues

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Abstract

A *class-III chitinase* promoter was isolated from *Lupinus albus.* The region 5′ to the coding sequence of the *IF3* gene was amplified by gene walking and sequenced. The proximal 2.0 kb sequence contains a predicted promoter site, including a TATA box, near the ATG start site. To test for minimal sequences needed for promoter activity, the region was restricted into fragments of 1.81, 1.51 and 1.13 kb and cloned into the pDM327 vector, upstream from the *bar-gus* fusion gene for Biolistic™ transformation. Transformation of lupin embryos, bean callus tissue, maize embryos and *Ornithogalum* callus demonstrated promoter activity for all fragments. In silico analysis identified putative *cis*-acting elements in the 1.81 kb fragment that could be important in controlling gene expression. Fungal elicitor activated-, wound-inducible- and ethylene responsive elements were present in the 1.51 kb fragment. Myb elements and CAAT boxes that regulate responses to environmental factors and modulate promoter efficiency were identified in the 1.81 kb fragment. The 1.51 and 1.81 kb fragments were inserted upstream of the *gus* gene into the pBI121 vector for *Agrobacterium tumefaciens* transformation of tobacco. Quantitative GUS assays indicated that the promoter fragments are functional *in planta* and inducible by defense-related signals, wounding, as well as chemical elicitation. All important elements essential for Bion inducibility are present on the shorter (1.51 kb) promoter fragment, but both 5′ distal and proximal *cis*-elements are required for full functionality. The *IF3* promoter is, thus, suitable for use in defense gene constructs prepared for the production of anthracnose resistant lupin.

Keywords

Biolistics Cis-elements Chitinase Lupin Pathogenesis-related Promoter Regulation

Introduction

Several species of lupins are cultivated in Mediterranean climate regions, since they can tolerate acidic and sandy soils and provide a crop rotation option for wheat (www.lupins.org) (Adhikari et al. 2013). Narrow-leafed lupin, *Lupinus angustifolius* is grown for the animal feed market as an alternative to soybean (Adhikari et al. 2013). *Lupinus albus* on the other hand is a species with a larger seed and higher protein content that is grown for the human health food market (Adhikari et al. 2009).

Lupin production is however challenged by anthracnose disease, which can cause yield losses of up to 100 % (Koch et al. 2002; Adhikari et al. 2013). Lupin anthracnose is caused by *Colletotrichum lupini* (previously classified as *C. gloeosporioides*, Nirenberg et al. 2002) which is able to infect both foliar tissues as well as pods of lupin species (Lotter and Berger 2005). Most accessions of *L. albus* are highly susceptible, and during the 1990s growers had to abandon production of this species in Western Australia and the Western Cape in South Africa due to the disease (Lotter and Berger 2005; Adhikari et al. 2009). Deployment of newly developed lines with increased resistance in Australian breeding programmes is hoped to result in a resurgence of production (Adhikari et al. 2013).

Current knowledge of the molecular responses of *L. albus* during anthracnose disease is very limited. Chitinases have been characterized as important plant defense enzymes in other plants (Grover 2012), and these represent a good starting point for the study of *L. albus* defenses. Chitinases hydrolyze the ß-1,4-linkage between N-acetylglucosamine residues of chitin, a structural polysaccharide in the cell wall of many fungi. Chitinases are carbohydrate active enzymes (CaZYs), and most plant chitinases are placed into two CaZY families, glycosyl hydrolase 18 and 19 (Collinge et al. 1993; Henrissat and Bairoch 1993; Grover 2012). They are pathogenesis-related (PR) proteins and are part of the defense arsenal of plants (Grover 2012). There are seven different classes, I to VII (Neuhaus 1999). Most of the class III chitinases occur extracellularly (Yeboah et al. 1998). Class III chitinases are classified as family 18 glycosyl hydolases (CAZypedia, http://www.cazy.org/GH18.html). Based on comparative nucleotide sequence analysis, Regalado et al. (2000) classified *IF3* as a class III basic (Chib1) chitinase.

Upon fungal infection, a number of chitinase genes are induced in the plant (Roby et al. 1990). Purified chitinase extracts restricted fungal growth in vitro (Broekaert et al. 1988; Mauch et al. 1988; Verberg and Huynh 1991). Furthermore, chitinase transgenic plants with demonstrated increased expression levels were less susceptible to certain pathogens (Broglie et al. 1991; Jach et al. 1995).

Regalado et al. (2000) reported that the *L. albus* genome contains only one class III *chitinase* gene, which they termed *IF3* (*Intracellular Fluid 3*), since the protein was detected in the apoplast. These authors demonstrated the expression of IF3 chitinase following infection with *Colletotrichum lupini*, suggesting that it is involved in plant defense, since there was an increase in the accumulation of *IF3* mRNA, as well as the IF3 protein.

The induction of chitinases following fungal infection (Roby et al. 1990; Broglie et al. 1991; Jach et al. 1995; Regalado et al. 2000) suggest that chitinase gene expression is initiated by an inducible promoter. Thus, it was decided to isolate the lupin *IF3* chitinase promoter to drive expression of genes that could potentially be used to enhance resistance of transgenic plants against fungal infection. The advantage of using an inducible promoter is that it would only be activated during anthracnose infection, and the protein would only be expressed when needed, resulting in a reduction in energy cost to the plant (Gurr and Rushton 2005).

The aim of this study was to isolate the *L. albus IF3* promoter, followed by the analysis of the isolated *IF3* DNA sequence for possible promoter sites and *cis*-acting controlling sequences (Azhakanandam et al. 2015). Subsequently, it was necessary to test for the minimal sequence needed to retain promoter activity, and to demonstrate that the promoter is functional *in planta* following biolistic transformation of a number of plant tissues and stable

Agrobacterium tumefaciens-mediated tobacco transformation with the promoter:*gus* constructs.

Materials and methods

Genome walking, cloning and sequencing of promoter constructs

DNA was extracted from leaf tissue of lupin seedlings using a DNeasy Plant Mini Kit (Qiagen, Venlo, Netherlands. Genome walking upstream from the 5′-end of the *IF3* gene was performed using the GenomeWalker™ kit (Takara, Clontech, Mountain View, CA, USA). DNA (2.5 µg) was restricted at 37 °C with *Dra*I, *EcoR*V, *Pvu*II and *Stu*I supplied with the GenomeWalker™ kit (Takara, Clontech, Mountain View, CA, USA), according to standard procedures (Sambrook et al. 1989). Ligations of each set of cleaned-up and restricted DNA to the GenomeWalker™ adaptors was subsequently performed.

Two gene-specific primers (GSP), one for the primary PCR reaction (GSP1) and one for the secondary PCR reaction (GSP2), were designed within the 5′ end of the *IF3* sequence based on the cDNA sequence deposited in Genbank Y16415: GSP1: 5′-CTTCCAGCACCACCACCAAGTGAG-3′ and GSP2: 5′-GCCAGCAGCATTGGACAACTTGAA-3′.

PCR amplification conditions included an initial denaturation step of 95 °C for 1 min. This was followed by 35 cycles with denaturation at 95 °C for 30 s and annealing at 68 °C for 6 min. A final extension step at 68 °C for 15 min was included. *Stu*I digests yielded a 4.2 kb band that was further analysed. PCR products were re-amplified using the Expand Long Distance Template *Taq* polymerase (Roche, Mannheim, Germany) and extracted from a 1 % (*w*/*v*) agarose gel, using the QIAquick Gel Extraction kit (Qiagen, Venlo, Netherlands, following electrophoresis of the PCR products.

PCR products were ligated into the pGEM-T Easy vector (Promega, Madison, WI, USA) and transformed into *Escherichia coli* DH5α competent cells. Isolation of plasmid DNA from *E. coli* was done using the Qiaprep Mini plasmid purification kit (Qiagen, Venlo, Netherlands). Sequencing of the pGEM-*Stu*I-4.2 bp (pGEM:4.2 kb) construct was by Microsynth (Balgach, Switzerland). The sequence data obtained for the 4.2 kb *IF3* promoter-containing fragment (GenBank accession number KP981368) was compared to known sequences in the GENBANK database (http://www.ncbi.nlm.nih.gov/Genbank/).

Identification of promoter sites and *Cis*-regulatory elements

The Berkeley Drosophila Genome Project (BDGP): Neural Network promoter prediction site was used to predict possible promoter sites, including the TATA boxes, within the isolated 4.2 kb fragment (http://www.fruitfly.org/seq_tools/promoter.html/).

In order to identify putative defense-related *cis*-elements in the promoter area of *IF3*, a plant database on *cis*-acting regulatory elements (PlantCARE, http://intra.psb.ugent.be:8080/PlantCARE; Lescot et al. 2002) was utilised. PlantPAN (http://PlantPan.mbc.nctu.edu.tw; Chang et al. 2008) and PLACE (http://www.dna.affrc.go.jp / PLACE; Higo et al. 1999) were also consulted. The 1.81 kb (−1800 bp) promoter sequence upstream of the predicted start codon (ATG) was chosen since most plant promoters display *cis*-elements within this region (New et al. 2015).

Cloning of promoter fragments into the pDM327 vector

The putative promoter fragments were cloned into the pDM327 vector (Kamo et al. 2000) for Biolistic™ transformation experiments to test for promoter activity in various plant tissues. The 2.18 kb *IF3* fragment was PCR re-amplified from pGEM: 4.2Promoter using primers designed to the 4.2 kb fragment. The sense primer (RePromP2: 5′-CAAATCTTTGAGCCTAACAGTATACAGAGATGAG-3′) was designed to match the promoter sequence. The antisense primer (RePromP1: 5′-GCGC**GGATCC**GTTTGTAGCTATATTCCAAGTTGT-3′) was designed so that a *BamH*I site (bold underlined) was incorporated at the 5′-end of the primer to facilitate cloning of the PCR product into the *BamH*I site of pDM327. The annealing step was at 60 °C for 90 s and the pGEM:4.2Promoter plasmid DNA was used as a template in this PCR reaction.

DNA fragments (2.18 kb) were gel extracted and cloned into the pGEM-T Easy vector, resulting in a clone termed pGEM: 2.2Promoter. Truncated promoter-containing fragments were released from the pGEM:2.2Promoter plasmid with different restriction enzyme combinations: *Nco*I and *BamH*I to yield a 1.817 kb fragment, *Nsi*I and *BamH*I to yield a 1.519 kb fragment, and *Bgl*II and *BamH*I to yield a 1.138 kb fragment. In all three cases the restricted DNAs were subjected to a Klenow DNA polymerase I large fragment fill-in step, before being restricted with *BamH*I.

In pDM327 the CaMV 35S promoter had been cloned into the vector upstream from the *bar-gus* translational fusion gene as a *Hind*III/*BamH*I fragment. This was substituted with the *IF3* promoter-containing fragments. The pDM327 was restricted with *Hind*III and subjected to a Klenow polymerase fill-in step before being restricted with the *BamH*I restriction enzyme to remove the CaMV 35S promoter fragment. The three *IF3* promoter-containing blunt end – *BamH*I fragments were then cloned separately into the pDM327 vector upstream from the *bar-gus* translational fusion gene. The resulting constructs pDM327:Prom1.8, pDM327:Prom1.5 and pDM327:Prom1.1 were tested for promoter activity using Biolistic™ transformation of plant tissue and staining for GUS activity.

Biolistic™ transformation of plant tissue to test for promoter activity

The QIAfilter Plasmid Mega kit (Qiagen, Venlo, Netherlands) was used for the preparation of pDM327neg, pDM327, pDM327:Prom1.8, pDM327:Prom1.5 and pDM327:Prom1.1 plasmid DNA. The Biolistic Particle Delivery System, model PDS-1000/He (Bio-Rad, Hercules, CA, USA) was used for transformation.

Ornithogalum (A2/ Rolou) callus (a South African *Ornithogalum* breeding line, a result from a cross between *O. dubium* and *O. thyrsoides*), lupin (*L. albus*) immature embryo tissue, bean (*Phaseolus vulgaris*) callus tissue and maize (*Zea mays*) immature embryos were prepared. The latter was obtained from Dr. M O'Kennedy, CSIR Biosciences, Pretoria, South Africa.

The pDM327:Prom1.1, pDM327:Prom1.5 and pDM327:Prom1.8 plasmid DNA was used for the Biolistic™ transformation of the plant tissue. The positive control used was the pDM327 plasmid DNA (contains the CaMV 35S promoter upstream from the *bar-gus* translational fusion gene), and the negative control was pDM327neg plasmid DNA (no promoter upstream from the *bar-gus* translational fusion gene). Biolistic™ transformation of plant tissue was performed in triplicate for each of the constructs using the protocol described in De Villiers et al. (2001).

After the bombardment of plant tissue, the material was placed in the dark at 26 °C for 2 days. Each plate was then stained with 1.0 mL of X-Gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronide name) stain solution. The stained tissues were incubated overnight in the dark at 37 °C. The numbers of blue spots appearing were recorded and results photographed.

Agrobacterium-mediated transformation of tobacco

The pBI121-neg construct was prepared by ex*cis*ing the CaMV promoter from pBI121 to serve as a negative control during promoter studies of transformed tobacco. pBI121 plasmid DNA was digested with *Bam*HI and *Hind*III, blunted with Klenow enzyme, the appropriate fragment recovered from an agarose gel using the QIAquick gel extraction kit (Qiagen, Venlo, Netherlands) and self-ligated using T4 DNA ligase according to standard protocols (Sambrook et al. 1989). The 1.51 kb and 1.81 kb promoter fragments were prepared from the respective pDM327 plasmids with *Nsi*I/*Bam*HI and *Nco*I/*Bam*HI, respectively, and ligated to the similarly prepared pBI121 vector. Ligation reactions were transformed into competent *E. coli* DH5α cells and positive transformants selected on LB agar plates containing 50 μg/mL kanamycin. Restriction enzyme analysis and PCR screening of putative transformants were performed to identify true recombinants. The Expand Long template PCR (Roche, Mannheim, Germany) system was used to amplify the promoter fragments from recombinant pBI121:Prom1.5 and pBI121:Prom1.8 constructs using the M13Rev (5′-CAG GAA ACA GCT ATG AC-3′) and GUS sequencing primers (5′-TCA CGG GTT GGG GTT TCT AC-3′). The products were separated by agarose gel electrophoresis and the bands of interest sequenced (Inqaba Biotech, Pretoria, South Africa).

Chemically competent *A. tumefaciens* LBA4404 cells were transformed with the different pBI121-constructs by freeze-thaw and selection on LB agar plates containing 50 μg/mL each of rifampicin and kanamycin. Several transformed colonies were obtained for each of the pBI121 constructs and PCR screening indicated the expected amplified products. One clone of each construct was selected for tobacco transformation. Sterile disks of in vitro propagated *Nicotiana tabacum* (cv. LA Burley) leaves were submerged under suspensions of *A. tumefaciens* LBA4404 transformants in Murashige and Skoog (MS) medium with an OD_{620} of 0.8, blotted dry and placed on MS-agar plates. After 48 h incubation in a growth room, the disks were subcultured onto regeneration medium (MS-agar containing 0.5 mg/L indole acetic acid, 1 mg/L benzyl adenine, 100 mg/L kanamycin and 250 mg/L cefotaxime). The leaf disks were subcultured to fresh regeneration medium at day 17 and day 32, where after individual shoots were transferred on day 44 to MS agar ($kanamycin^{100}$ $cefotaxime^{250}$) for rooting. Plantlets were maintained on MS agar ($kanamycin^{100}$ $Cefotaxime^{250}$), with sub-culturing every 1 to 2 months, until contamination by *A. tumefaciens* had been eliminated, where after cefotaxime was omitted from the culture medium.

Plant genomic DNA was isolated from in vitro leaf disks using the CTAB extraction method and isopropanol precipitation (Murray and Thompson 1980). PCR screening using the M13Rev, NOSPolyA (5′-GAT AAT CAT CGC AAG ACC GGC AAC-3′) and Gus sequencing primers and the *nptII* primers (NPTII-L (5′-GAG GCT ATT CGG CTA TGA CTG-3′) and NPTII-R (5′-ATC GGG AGC GGC GAT ACC GTA-3′)) were performed according to standard protocols. Positive controls containing plasmid DNA of the respective pBI121 constructs and negative water controls were included.

Leaf disks from each in vitro transgenic tobacco line were histochemically stained for GUS activity in GUS staining buffer [0.521 mg/mL X-Gluc in 100 mM $NaPO_4$ buffer, pH 7.0;

10 mM EDTA; 0.1 % Triton X-100; 0.5 mM K-ferricyanide; 0.5 mM K-ferrocyanide] and the chlorophyll bleached with 70 % ethanol. The leaf disks were screened for the presence of dark blue spots.

GUS expression assays of transgenic tobacco induced by elicitors

Leaf disks were treated with various elicitors to induce expression of GUS by the 1.51 and 1.81 kb *IF3* promoter fragments. These included Bion (acibenzolar-*S*-methyl benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester) (200 μM, Syngenta, South Africa), sodium salicylate (400 μM, Sigma Aldrich, Germany) and ethepon (3.3 mM, Sigma Aldrich, Germany) in ¼ strength MS medium (pH 5.8), wounding by pinching each leaf disk four times with a tweezer, and ¼ × MS (pH 5.8) as control. Ten mm diameter leaf disks were prepared from three biological replicates (3 T0 clones of each event), 40 disks per plant, of greenhouse grown plants of a single selected transgenic line of each construct. The discs were floated on top of the respective elicitor solutions, with the abaxial side of the leaf facing down, at 25 °C for 24 h or 48 h with continuous illumination.

Ten mm leaf disks were homogenised with carborundum C-400 and extracted with extraction buffer (1:2 m/v) (Jefferson et al. 1987) for 1 h on ice. The supernatant was cleared from plant debris by centrifugation and the protein concentration determined (Bradford 1976). Supernatants (100 μL) were assayed for GUS activity by mixing it with 400 μL assay buffer in a total volume of 500 μL. Reactions were incubated at 37 °C, and 100 μL aliquots removed at 40, 80 and 120 min and added to 400 μL stop buffer (0.2 M Na_2CO_3). Fluorescence of these samples was measured in duplicate with a Fluoroskan Ascent FL microplate reader (Thermo Fischer Scientific, Waltham, MA USA) (excitation 355 nm, emission 460 nm) in black microwell plates.

A standard curve of 4-methyl umbelliferone (4-MU) ranging from 0 to 10 μM in stop buffer was constructed to determine the linear range of the fluorometric assay for GUS. The increase in relative fluorescence (RF) against time (min) was determined and the GUS activity values were calculated and expressed as pmol 4-MU released/ min/ mg protein. Statistical analyses were performed using the Fisher's protected least significant difference test using the statistical program GenStat (2011).

Results

Cloning and sequencing of the 4.2 kb promoter region of lupin *IF3* chitinase

The ~4.2 kb insert in the plasmid pGEM-4.2Promoter (pGEM-*Stu*I-4.2 kb) cloned from *L. albus* by genome walking was sequenced (GenBank accession number KP981368). As expected, the 3′-end of the sequence corresponded to the 5′-end of the *L. albus* chitinase III gene reported in Regalado et al. (2000), from the ATG to the gene-specific primer used for the secondary PCR (GSP2) (Fig. S1). BLASTN/X analysis of the 4.2 kb sequence against the GenBank database indicated that it comprised two sections – a 5′ section of approximately 2.2 kb that contained open reading frames. It is likely that the 5′ section containing ORFs code for exon(s) of part of a gene upstream of the *IF3* gene in *L. albus*, and was not further investigated in this study. BLASTN/X analysis of the 3′ end of the 4.2 kb fragment (nucleotides 2100–3960) showed no significant nucleotide identity to sequences in the Genbank database, lacked any ORFs, and was, thus, likely to contain the promoter and transcriptional regulatory regions of the lupin *IF3* chitinase gene.

Bioinformatics analysis (http://www.fruitfly.org/seq_tools/promoter.html/) was used to predict core promoter elements to assist with the choice of regions for nested deletion analysis. Three putative core promoter regions (−1407 to −1357, −937 to −887 and −41 to −90) were predicted on the 3′ region that did not contain ORFs, with the most likely core promoter region closest to the ATG (position +1 to +3) of the chitinase gene (Porto et al. 2014) (i.e. corresponding to positions −90 to −41). These regions were therefore included for nested promoter deletion analysis (Fig. S2).

Cloning of nested 1.81, 1.51 and 1.13 kb lupin *IF3* chitinase promoter-GUS fusions

The 2.18 kb *IF3* promoter-containing fragment was re-amplified from pGEM:4.2Promoter using primers RePromP2 and RePromP1, designed to the 5′- and 3′-ends, and cloned into the pGEM-T Easy vector to produce the pGEM:2.2Promoter. Restriction enzyme (*EcoR*I) digestion of the recombinants yielded the expected 2.18 kb promoter-containing fragment. The pGEM:2.2Promoter clone was sequenced and aligned to the pGEM:4.2Promoter sequence, and was found to be identical (data not shown).

To identify functional promoter regions within the isolated putative promoter fragment, deletion fragments were fused to the *gus* reporter gene to determine the minimal sequence needed to retain promoter activity (Bustos et al. 1989). Following this approach, restriction digests with *Nco*I/*BamH*I, *Nsi*I/*BamH*I and *Bgl*II/*BamH*I (Fig. S3) yielded the promoter-containing fragments of 1.81 kb, 1.51 kb and 1.13 kb, respectively, as shown in Fig. S2.

To create the gene constructs for use of ß-glucuronidase (GUS) as reporter gene (Thomasset et al. 1996), the three fragments of the *IF3* promoter-containing sequences were cloned separately upstream from the *bar-gus* translational fusion gene in the pDM327 vector by replacement of the CaMV 35S promoter (Fig. S4). The correct clones were confirmed by *Sal*I restriction digests: clones pDM327:Prom1.1, pDM327:Prom1.5 and pDM327:Prom1.8 yielded the expected 1.46 kb / 1.84 kb / 2.14 kb fragments containing the respective 1.13 kb / 1.51 kb / 1.81 kb promoter-containing fragments (data not shown).

Transient transformation of dicot and monocot plant tissues with nested lupin *IF3* chitinase promoter-GUS fusions

Biolistic™ transformation has proven to be successful in monocotyledonous plants. It yields rapid results for transient expression and the high level of ß-glucuronidase (GUS / *uid*A) expression enables rapid histochemical screening of transformants for transgene activity (Cornejo et al. 1993). Thus, the information needed, i.e. whether the *IF3* promoter-containing DNA fragment isolated from *L. albus* has promoter activity, and whether promoter activity could be observed in both dicots and monocots, could be obtained rapidly using Biolistic™ transformation.

The pDM327:Prom1.1, pDM327:Prom1.5 and pDM327:Prom1.8 plasmid DNA was used for the Biolistic™ transformation of the *Ornithogalum*- and bean callus tissues, as well as immature maize - and lupin embryos. The positive control used in the experiment was the pDM327 plasmid DNA (containing the CaMV 35S promoter upstream from the *bar-gus* translational fusion gene), and the negative control was pDM327neg plasmid DNA (no promoter upstream from the *bar-gus* translational fusion gene). After histochemical staining, following the Biolistic™ transformation of the various plant tissues, GUS-positive spots were

observed and counted. Figure 1 shows the results obtained with the 1.13 kb *IF3* promoter-containing fragment, with summarized data for all the fragments in Table 1.

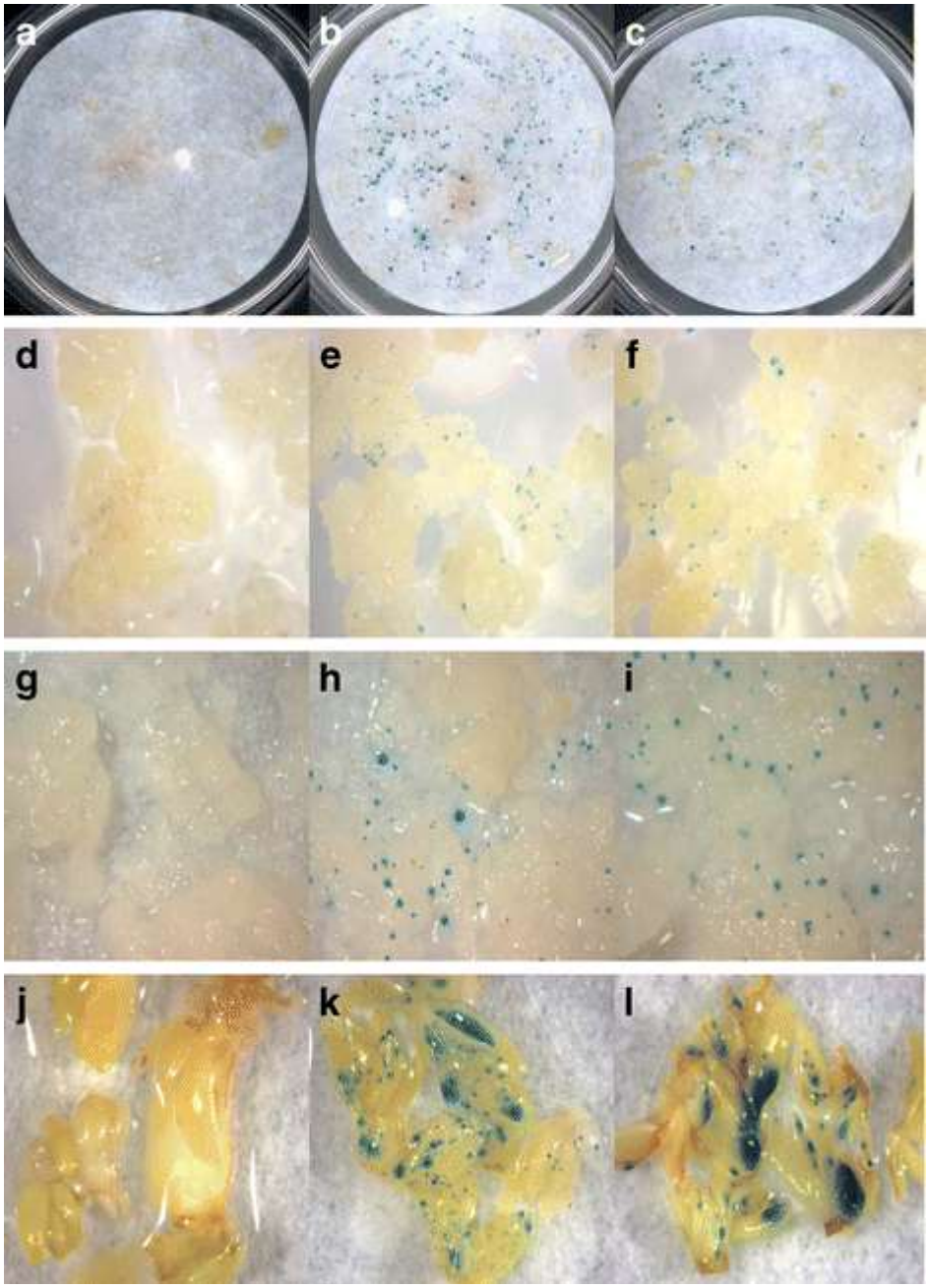

Fig. 1 : Transient GUS expression following Biolistic™ transformation of *Ornithogalum* callus tissue, maize immature embryo tissue, bean callus cultures and immature lupin embryo tissue. **a**: *Ornithogalum* callus tissue using the pDM327 neg construct (negative control), **b**: using the pDM327 construct (positive control) under the control of the Cauliflower Mosaic Virus (CaMV) 35S promoter and **c**: using the pDM327:Prom 1.1 construct containing the 1.13 kb *class-III chitinase* (*IF3*) promoter-containing fragment isolated from *Lupinus albus.* **d**: Maize immature embryo tissue using the pDM327 neg construct (negative control), **e**: the pDM327 construct (positive control) under the control of the CaMV 35S promoter and **f**: using the pDM327:Prom 1.1 construct. **g:** Bean callus cultures using the pDM327neg construct (negative control), **h**: the pDM327 construct (positive control) under the control of CaMV 35S promoter and **i**: using the pDM327:Prom 1.1 construct. **j**: Lupin immature embryo tissue using the pDM327 neg construct (negative control), **k**: the pDM327 construct (positive control) under control of the CaMV 35S promoter and **l**: using the pDM327:Prom 1.1 construct

Table 1: GUS activity results from transient Biolistic™ transformation of the *Ornithogalum* - and bean callus as well as maize and lupin immature embryos using the nested lupin *IF3* (*chitinase III*) promoter-GUS fusions

Construct	Promoter fragment	Average number of blue spots per treatment			
		***Ornithogalum* callus**	**Bean callus**	**Immature maize embryos**	**Immature lupin embryos**
pDM327neg	None	0	0	0	0
pDM327	CaMV35S	272 ± 157	311 ± 93	397 ± 136	Medium*
pDM327:Prom1.1	1.13 kb *if3*	208 ± 105	552 ± 243	220 ± 14	Medium*
pDM327:Prom1.5	1.51 kb *if3*	136 ± 32	468 ± 266	284 ± 14	High*
pDM327:Prom1.8	1.81 kb *if3*	266 ± 94	154 ± 81	81 ± 37	Medium*

*****Relative GUS activity per treatment was visually assessed since the lupin tissue stained blue with large spots due to the high level of GUS expression. "Medium" indicates that approximately 50 % of plant tissue had stained blue, while "high" indicates that between 50–75 %, of plant tissue had stained blue

Expression of all three lupin derived *IF3*-promoter: GUS reporter gene constructs was observed in the two legume dicots (immature lupin embryos and bean callus). In addition expression was also successful in the two monocots (maize embryos and *Ornithogalum* callus) tissues, indicating that the *IF3* promoter is functional in both types of plants. From Table 1 no apparent differences in the GUS expression driven by the three promoter fragments can be seen. This was due to the high level of variability in the number of blue spots obtained between replicate bombardment experiments for each of the promoter-containing fragments. This resulted from the fact that the number of cells applied to each plate was not easy to calculate and varied from plate to plate, and from experiment to experiment. As expected, GUS activity was observed in all tissues with the positive control pDM327 (CaMV 35S promoter), whereas no GUS activity was observed for the pDM327 (negative) construct in which no promoter was present upstream from the *bar-gus* translational fusion gene (Table 1).

Analysis of the *IF3* promoter fragment for the presence of *Cis*-acting regulatory elements

In silico analyses (Berkeley Drosophila Genome Project (BDGP) Neural Network promoter prediction site (http://www.fruitfly.org/seq_tools/promoter.html) identified the most plausible core promoter sequence containing the TATA box and putative transcription start site (positions −41 to −90 and −50, relative to the translational start site). The score value of the prediction was 0.88. Moreover, the PlantCARE database, http://intra.psb.ugent.be:8080/PlantCARE, also identified the TATA-box of the *IF3* promoter in the core promoter region at position −30 from the transcription start site. The upstream *cis*-acting elements are of extreme importance in controlling promoter activity and, thus, regulating gene expression. In order to better understand the architecture of the *IF3* promoter-containing fragment isolated from *L. albus*, in silico analyses were performed in order to identify putative *cis*-acting regulatory elements that could be important in the control of *IF3* gene expression. The *IF3* promoter sequence with the putative *cis*-elements identified is shown in Fig. S1. The summarized data in Table 2 indicate putative defense-related *cis*-elements found in the upstream region up to −1.8 kb.

Table 2: The *cis*-acting elements identified in the *IF3* (*class-III chitinase*) promoter-containing fragment isolated from *Lupinus albus* using the PlantCARE database (Lescot et al. 2002) at: http://intra.psb.ugent.be:8080/PlantCARE

Position	Strand	Sequence	Present in fragments
W1-box			
-1510	+	TTGAcc	1.81 and 1.51 kb
-1170	-	TTGAcc	1.81, 1.51 and 1.13 kb
-1097	-	TTGAcc	1.81, 1.51 and 1.13 kb
Elicitor response (ELI) - box3			
-1646	+	AAACaaatt	1.81 kb
-926	-	AAACcaatt	1.81, 1.51 and 1.13 kb
-861	-	AAACctatt	1.81, 1.51 and 1.13 kb
-853	-	AAACcaata	1.81, 1.51 and 1.13 kb
-46	+	AAACcaata	1.81, 1.51 and 1.13 kb
WUN-motif			
-1522	+	cAATTtcta	1.81 kb
-1552	+	tGATTtcta	1.81 kb
-1727	-	aCATTtcaa	1.81 and 1.51 kb
-934	+	tAATTgcat	1.81, 1.51 and 1.13 kb
-674	-	tCATTtcat	1.81, 1.51 and 1.13 kb
-1071	-	cAATTacat	1.81, 1.51 and 1.13 kb
-849	+	tAATTtcac	1.81, 1.51 and 1.13 kb
-861	+	aTATTgcga	1.81, 1.51 and 1.13 kb
-532	+	cCATTtccc	1.81, 1.51 and 1.13 kb
-1433	+	aCATTtcaa	1.81, 1.51 and 1.13 kb
-431	+	tGATTtcat	1.81, 1.51 and 1.13 kb
-1497	+	aAATTccta	1.81, 1.51 and 1.13 kb
-522	+	cAATTacaa	1.81, 1.51 and 1.13 kb
-495	-	aAATTgcta	1.81, 1.51 and 1.13 kb
-1171	+	cAATTtcta	1.81, 1.51 and 1.13 kb
-879	+	tAATTtctt	1.81, 1.51 and 1.13 kb
-1531	+	tCATTcctt	1.81, 1.51 and 1.13 kb
-546	+	tTATTccct	1.81, 1.51 and 1.13 kb
88	-	tGATTgcca	1.81, 1.51 and 1.13 kb
Ethylene-responsive element (ERE)			
-1764	+	ATTTctaa	1.81 kb
-123	+	ATTTctaa	1.81 kb
-1489	-	ATTTcaat	1.81 and 1.51 kb
-1089	-	ATTTcata	1.81, 1.51 and 1.13 kb
-1611	-	ATTTaaaa	1.81, 1.51 and 1.13 kb
-497	+	ATTTcaat	1.81, 1.51 and 1.13 kb
-153	+	ATTTctaa	1.81, 1.51 and 1.13 kb
MYB binding site			
-1543	+	CGGTta	1.81 kb
-595	+	CGGTca	1.81 and 1.13 kb

To verify the functional significance of these in silico predicted *cis*-elements, the inducibility of the promoter activity in response to elicitation with various treatments was investigated by means of stable transformation of the promoter-GUS constructs in the tobacco background.

Transformation of tobacco with lupin *IF3* promoter-GUS fusion genes, and assays for elicitor induction

The 1.51 kb and 1.81 kb promoter-GUS fusion constructs for stable transformation of tobacco was successfully constructed, namely pBI121:Prom1.5 and pBI121:Prom1.8. The pBI121-neg colony was screened with PCR to distinguish it from native pBI121 plasmid using combinations of PCR primers (M13Rev and GUS sequencing or NOS-PolyA primers) and the expected PCR products were obtained. Restriction enzyme analysis of the other two promoter-GUS fusion constructs, using *Nco*I, *Pst*I and *Hind*III restriction enzymes, also yielded the expected restriction patterns (data not shown). Sequencing of PCR amplified fragments of the 1.81 and 1.51 kb promoter-GUS fusion constructs yielded the expected sequences (data not shown).

After *Agrobacterium*-mediated transformation of tobacco leaf disks, a total of 45 individual T0 transgenic plants, containing the three different constructs, were clonally multiplied and analysed. Leaf disks from the individual transgenic plants were screened with PCR and GUS staining for the presence and expression of the expected promoter-GUS fusion construct and the *nptII* gene conferring kanamycin resistance to the transgenic tobacco (data not shown).

Representative lines, positive for PCR and GUS staining, i.e. pBI121-neg, pBI121:Prom1.5 and pBI121:Prom1.8, were selected for studies to evaluate the elicitor-responsiveness of the two promoter fragments through activity assays of the GUS reporter gene. Figure 2 represents the average GUS activity in transgenic lines after 48 h treatment with different elicitors, grouped according to the elicitor treatment. pBI-neg transformed tobacco (Neg) contained no GUS activity, as expected. The different elicitors had different effects on the GUS activities. Wounding elicited much weaker responses compared to Bion, Ethepon and sodium salicylate (SA). There was no significant difference in GUS activity between the wounded and control samples, probably due to wounding-related injury when punching the leaf disks. The trend of eliciting activity of the five treatments follows a decreasing order of
Bion > Ethepon > SA > Wounding > Control.

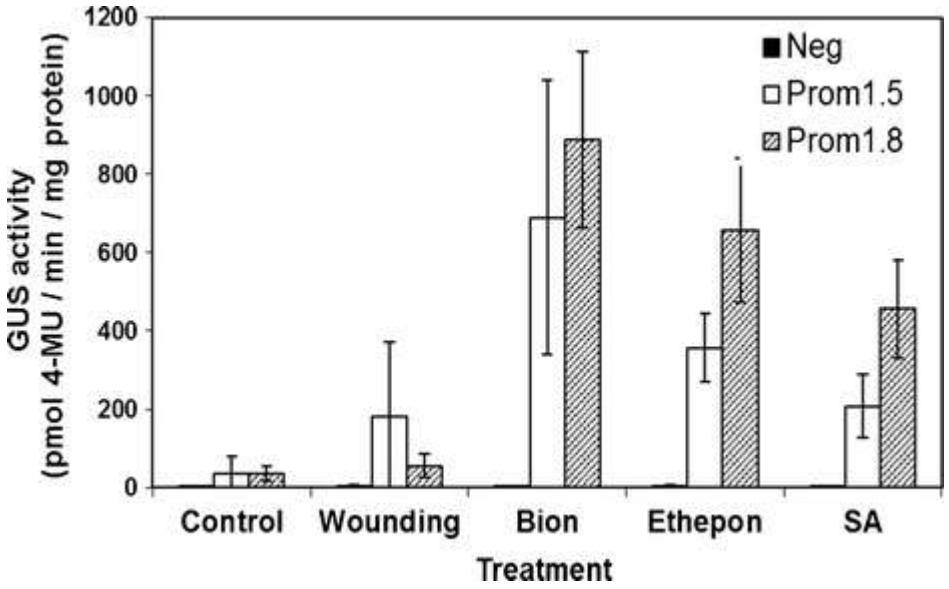

Fig. 2: GUS activity measured in leaf disks of T0 transgenic tobacco lines treated with elicitors for 48 h. Activity is presented as pmol 4-MU released/min/mg protein. Values represent averages of triplicate biological replicates. Legend: Control (MS medium); Wounding; Bion (200 μM); Ethepon (3.3 mM); SA: Salicylic acid (sodium salicylate) (400 μM). *Black bars*: negative control, pBI121-neg; *White bars*: Prom1.5; *Grey bars*: Prom1.8. The *error bars* indicate the standard deviation of three biological replicates of each *line*

Except for wounding, all treatments (Bion, Ethepon and SA), showed statistically significant induction of GUS expression by the 1.5 and 1.8 kb promoter fragments at the 95 % confidence level, when compared to the control untreated samples. Furthermore, the 1.8 kb promoter-GUS fusion plants showed higher GUS activity than the 1.5 kb promoter. The average GUS activity in Prom1.5 and Prom1.8 transgenic lines was also compared at an earlier time point, 24 h after Bion treatment (graph not shown). The induced levels of both the pBI121:Prom1.5 and pBI121:Prom1.8 transformants again showed statistically significant induction of GUS expression at the 95 % confidence level, when compared to the control untreated samples. When the induced GUS activity of the Prom1.8 fusion was compared to that of Prom1.5, there was no statistically significant difference at 24 h. These result, therefore, indicate that all the important elements essential for Bion inducibility are present on the shorter (1.51 kb) promoter fragment, but that elements found in the 300 bp region between 1.5 and 1.8 kb play an additional modulating role.

Discussion

Promoter identification and – architecture

A fragment of approximately 4.2 kb upstream of the *L. albus IF3* gene was amplified by 5′ gene walking. The sequence proximal to the *IF3* coding sequence was compared to known sequences in the Genbank database and exhibited no significant homology. In silico analysis predicted an eukaryotic promoter site, including a TATA box, within 30 nt from the ATG start site. In order to test for the minimal sequence needed to retain promoter activity, the 4.2 kb promoter-containing fragment was restricted into three smaller fragments of 1.8, 1.5 and 1.1 kb, which were each cloned separately into the pDM327 vector upstream from the *bar-gus* translational fusion gene for transient Biolistic™ transformation.

The CaMV 35S promoter used in the positive control vector pDM327 has been shown to be active after biolistic transformation of dicots (Odell et al. 1985), the legume soybean, cereals (maize, wheat)(Wang et al. 1988), and non-cereal monocots (Kamo et al. 2000). Our positive results with the CaMV 35S promoter in a range of legume and monocot tissues (Fig. 1) are consistent with these previous reports. Transient GUS expression was observed in both the monocotyledonous (maize and *Ornithogalum*) tissues and the dicotyledonous (bean and lupin) tissues, indicating that the *IF3* promoter is functional and that the cellular environment in both types of plants is conducive to transcription of the GUS gene. This is in agreement with Regalado et al. (2000) who reported a basal level of gene expression in non-stimulated *L. albus* tissues. Results for *Ornithogalum* and bean callus tissue, as well as immature embryos tissues of maize and lupin, showed that the number of transient transformants obtained per bombardment was reasonably high, compared to the results obtained with the 35S CaMV promoter construct (pDM327). In addition to constitutive activity of CaMV 35S promoter observed in stably transformed plants, activity after biolistic transformation may reflect induction by wounding caused by the particle penetration of cells. This may explain partially the expression after biolistics of the IF3 promoter-constructs in our study (Fig. 1), since Regalado et al. (2000) and our study (Fig. 2) showed wounded-induced expression, although lower than biotic stressors.

It can also be assumed that all *cis*-acting elements needed for induction of the *IF3* promoter, and, thus, transient GUS expression in all tissues tested, are present on the 1.13 kb *IF3* promoter-containing fragment. However, no conclusions could be made as to which of the three *IF3* promoter-containing fragments was the most efficient in stimulating expression of the *gus* reporter gene.

The TATA sequence alone is usually unable to activate transcription significantly in vivo, and additional *cis*-elements upstream are required to drive expression from these sites (Azhakanandam et al. 2015). The positive GUS staining suggests the presence of abundant endogenous promoter-binding factors that recognises their *cis*-binding sites in the isolated promoter to activate *gus* reporter gene expression. This prompted the investigation of the occurrence of various *cis*-acting elements in its architecture.

Analysis of a series of 5′ deletions of the acidic *class-III chitinase* promoter from Arabidopsis indicated that the proximal 192 bp upstream from the transcription start site was sufficient to establish both constitutive and inducible expression (Samac and Shah 1991). Elements further upstream were responsible for the quantitative expression of the gene and included both positive and negative regulatory elements. The study emphasised the importance of deletion studies in order to elucidate the functional organisation of a promoter fragment.

Since defense-related *chitinase* gene expression has been reported to be induced by various factors such as elicitors, wounding, SA and pathogen attack (Legrand et al. 1987; Ernst et al. 1992; Margis-Pinheiro et al. 1993; Graham and Sticklen 1994; Lawton et al. 1994), only the putative *cis*-acting elements identified in the 1.81 kb *IF3* promoter-containing fragment that have been reported in the literature to be essential for induction by elicitors, wounding, ethylene and pathogen attack, have been annotated in Fig. S1 and summarised in Table 2.

The Box-W1 *cis*-acting element, present in the promoters of parsley *pathogenesis related protein 1* (*PR-1*) genes, has been shown to regulate the transcription of the *PR-1* gene in response to a fungal oligopeptide elicitor (Rushton et al. 1996). The sequence (T)TGAC(C) was identified as the *cis*-acting element responsible for the fungal elicitor activation of the

PR-1 gene, since mutations that disrupted this sequence resulted in a loss of function. Three putative Box-W1 *cis*-acting elements were present in the *IF3* promoter-containing fragment. All three of these putative fungal elicitor responsive elements were present in the 1.81 and 1.51 kb *IF3* promoter-containing fragments, while two of them were present in the 1.13 kb *IF3* promoter-containing fragment.

The ELI-box3 *cis*-acting element has also been reported to be an elicitor responsive element (Ohl et al. 1990; Pastuglia et al. 1997). The *phenylalanine ammonia-lyase* (*PAL*) promoter from Arabidopsis contains two ELI-box3 regulatory elements reported to contain elicitor inducible activity (Ohl et al. 1990), and these elements have also been identified in a *PAL* gene from parsley (Lois et al. 1989). The ELI-box3 element is also present in the promoter of an S gene family receptor-like kinase (SRK) in *Brassicca oleracea* (Pastuglia et al. 1997). Five putative ELI-box3 *cis*-acting elements were present in the *IF3* promoter-containing fragment. All five of these putative fungal elicitor responsive elements were present in the 1.81 kb *IF3* promoter-containing fragment, while four of them were present in the 1.51 and 1.13 kb *IF3* promoter-containing fragments.

The WUN-motif has been identified as a wound-inducible *cis*-acting element (Matton et al. 1993; Pastuglia et al. 1997; Kaothien et al. 2000). The *sth-2* promoter of the *sth-2* pathogenesis-related gene in potato was reported to contain the WUN-motif (Matton et al. 1993) that has been reported to regulate the wound-inducible activity of the *wun1* and *wun2* genes from potato (Siebertz et al. 1989; Stanford et al. 1989). The WUN-motif was also present in the promoter of a *srk* gene in *Brassicca oleracea* (Pastuglia et al. 1997). Nineteen putative WUN-motif *cis*-acting elements were present in the *IF3* promoter-containing fragment. All 19 of these putative wound responsive elements were present in the 1.81 kb *IF3* promoter-containing fragment, 17 of them in the 1.51 kb *IF3* promoter-containing fragment and 16 of them in the 1.13 kb *IF3* promoter-containing fragments.

The expression of several plant chitinases have been shown to be induced by ethylene (Broglie et al. 1986; Memelink et al. 1990). Ethylene levels usually increase in response to stresses such as wounding and pathogen attack (Samac and Shah 1991). The ERE *cis*-acting element has been identified as an ethylene-responsive element (Itzhaki et al. 1994). Seven putative ERE *cis*-acting elements were present in the *IF3* promoter-containing fragment. All seven of these putative wound responsive elements were present in the 1.81 kb *IF3* promoter-containing fragment, while five of them were present in the 1.51 kb *IF3* promoter-containing fragment, and four of them in the 1.13 kb *IF3* promoter-containing fragment.

Wounding occurred during the Biolistic™ transformation of plant tissues in which the *IF3* promoter-containing fragments were tested for promoter activity, using transient GUS expression as indicator. Thus, it is hypothesised that due to the inducibility of the *IF3* promoter, that *gus* gene expression was activated as a result of the putative wound-inducible (WUN-motif) and ethylene responsive element (ERE) *cis*-acting elements. These data also show that most of these wound-inducible regulatory elements are present in the 1.13 kb *IF3* promoter-containing fragment. This could explain why this fragment was just as efficient in the activation of *gus* reporter gene, resulting in levels of transient GUS expression similar to that obtained with the 1.51 and 1.81 kb *IF3* promoter-containing fragments cloned upstream from the *bar-gus* translational fusion gene in pDM327.

Included in the elements listed in Table 2, are two putative Myb transcription factor binding site regulatory *cis*-acting elements, found to be present in the 1.81 and one in the 1.13 kb *IF3*

promoter-containing fragments respectively. Myb factors regulate the transcription of several plant genes in response to various environmental factors, including elicitors and wounding (Jin and Martin 2000; Sugimoto et al. 2000). The Myb sites are often located upstream from pathogen-inducible genes (Rushton and Somssich 1998).

The CAAT box element (consensus GCCCAATCT) plays an important role in the determination of promoter efficiency and several were also identified within the *IF3* promoter-containing fragments (data not shown). However, since these are not associated with inducibility of defense genes they have not been indicated in the analysis.

These in silico analyses indicate that most of the putative fungal elicitor activated *cis*-acting elements are present in the 1.13 kb *IF3* promoter-containing fragment and supports the hypothesis that all regulatory elements needed for the activation of the *IF3* gene promoter are located within the first 1.13 kb fragment upstream from the initiation codon of the *IF3* gene.

Promoter inducibility

The applicability of promoter-reporter gene fusions has been shown previously in transgenic tobacco transformed with a chimeric 1.7 kb fragment containing the bean *chitinase 5B* gene promoter and the *gus* gene. This promoter was transiently activated after fungal attack and the greatest induction of GUS expression was observed in and around the site of fungal infection (Roby et al. 1990). Following fungal infection, the increase in GUS activity reached a maximun at 48 h post infection and paralleled the increase in endogenous tobacco chitinase activity.

Here the GUS fusion gene concept was used to investigate the responsiveness of the *IF3* promotor deletions to chemical elicitation with Bion (acibenzolar-*S*-methyl benzo-(1,2,3)-thiadiazole-7-carbothioic acid *S*-methyl ester or BTH), signal molecules (SA and ethylene) and wounding. Exogenous application of chemicals such as SA, Bion and INA (2,6-dichloroisonicotinic acid) has been shown to activate the plant's natural immune responses (Ward et al. 1991). Bion is a water dispersible chemical used during plant cultivation, which elicits systemic acquired resistance (SAR) and stimulates the natural defense mechanisms of various plants against plant diseases to provide protection against damage caused by undesired microbes. Bion is a functional analog of SA because it induces the expression of known SA-responsive genes, but it acts independently of SA perception and biosynthesis (Kessmann et al. 1993; Friedrich et al. 1996), suggesting that it interacts with biological targets operating downstream from these steps.

To verify the functional significance of these in silico predicted *cis*-elements, the inducibility of the promoter activity in response to elicitation with various treatments was investigated at 24 h and 48 h post teatment by means of stable transformation of the promoter-GUS constructs in the heterologous tobacco background (Fig. 2). Promoter activation by, and the consequent inducibility of the GUS reporter gene, confirm that the *IF3* promoter fragment isolated from lupin is functional *in planta* and that the promoter is inducible to different extents by different elicitors. Notwithstanding biological variability, collection of quantitative promoter expression data indicated that both the 1.51 kb and 1.81 kb fragments of the promoter are highly inducible by Bion, but also, to a lower extent, responsive towards SA, ethylene and wounding. This might be an indication that both 5′ distal and proximal *cis*-acting regulator elements are required for full functionality of the promoter (Zheng et al. 1993).

Conclusion

In summary, the *L. albus IF3* promoter contains all the properties to allow its associated gene and the encoded class III chitinase to function as an inducible pathogenesis-related protein in the defense arsenal of lupin. As such, the *IF3* promoter would also be suitable to drive expression of genes that could potentially be used to enhance fungal resistance of transgenic plants. Moreover, the *IF3* promoter-containing fragment:*gus* chimeric genes produced in this study are suitable tools to study the cellular and molecular mechanisms of the activation of the host defense system in lupin as well as other legume crops during pathogen attack.

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Compliance with ethical standards

Conflict of interest

The authors declare no competing interests.

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Supplementary material

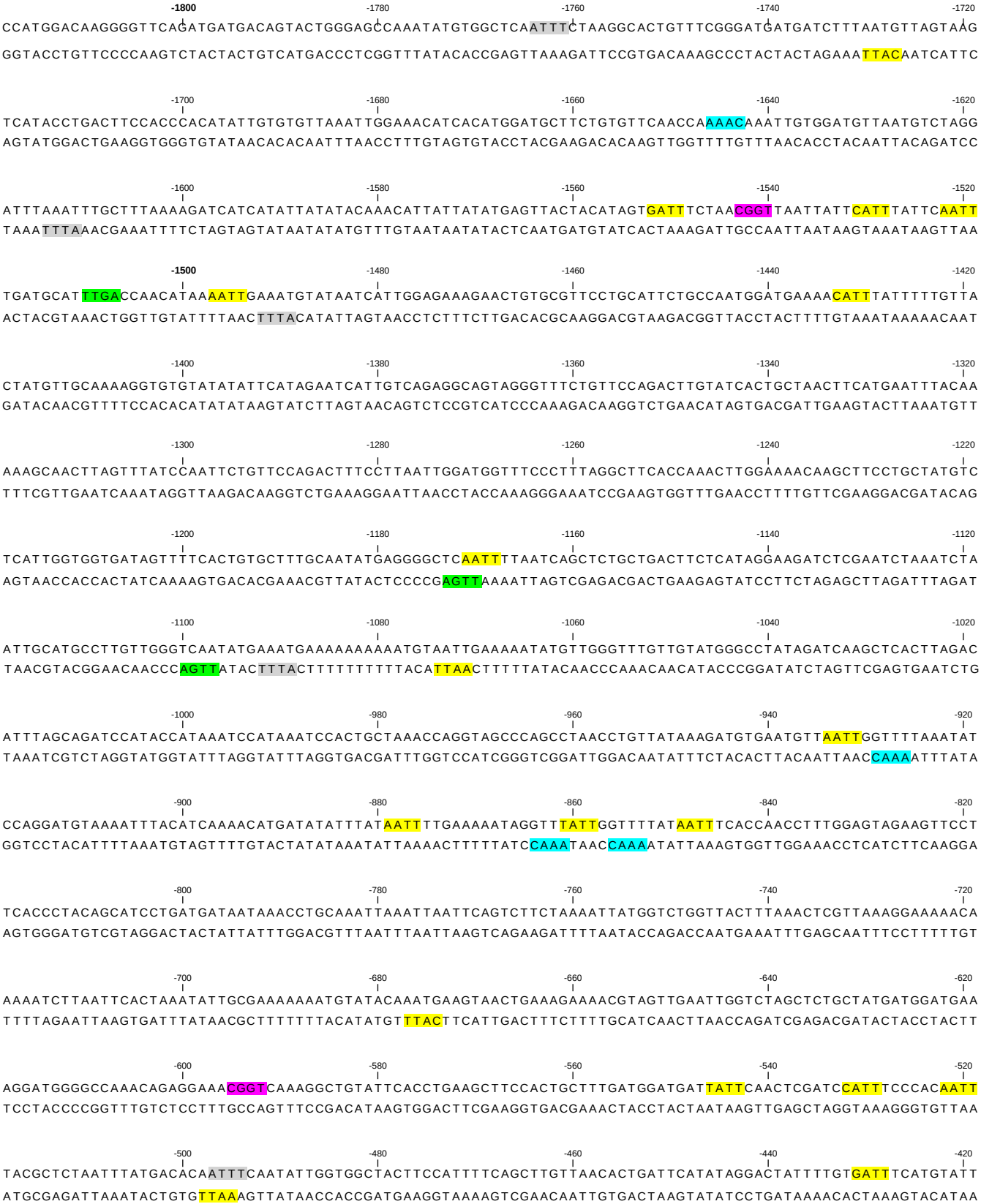

Figure S1. The complete sequence of the 1.818 kb *class-III chitinase* (*if3*) promoter-containing fragment isolated from *Lupinus albus* (corresponding to the 3' end of the 4.2 kb sequence deposited on Genbank Acc# KP981368).

In silico analyses (Berkeley Drosophila Genome Project (BDGP) Neural Network promoter prediction site - http://www.fruitfly.org/seq_tools/promoter.html) identified the most likely core promoter sequence within the fragment (red text, -41 to -90 nt) with TATA box underlined) and annotated the transcription start site (A) and translation starts site (ATG). The promoter prediction score was 0.89. Also indicated are the *L. albus if3* gene sequence (brown text) and the primer designed for Genome Walking (green text). Putative *cis*-elements identified using PlantCARE (http://intra.psb.ugent.be:8080/PlantCARE; Lescot *et al.*, 2002) are indicated with the following colour codes:

W1-box
Elicitor response (ELI) - box3
WUN-motif
Ethylene-responsive element (ERE
MYB binding site

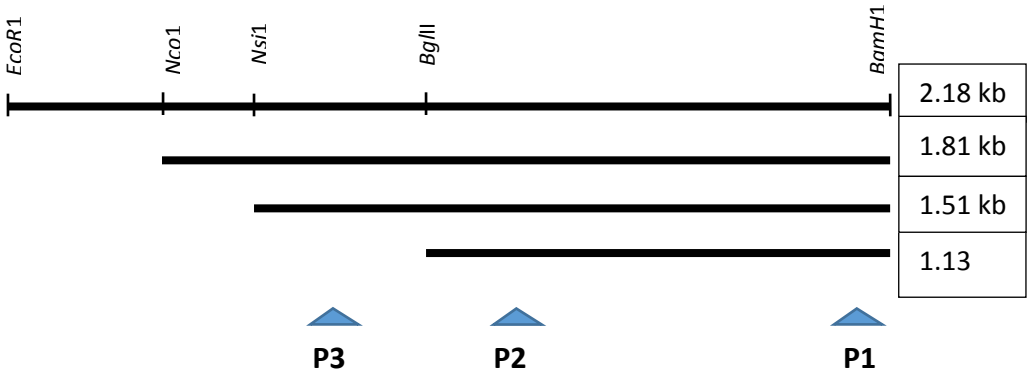

Figure S2 A graphic representation of the 3′ section of the 4.2 kb *class-III chitinase* (*IF3*) promoter-containing fragment isolated from *Lupinus albus*. Following promoter prediction analysis, the 2.18 kb fragment obtained from the pGEM:2.2Promoter was cut with restriction enzymes to yield 1.81 kb and 1.51 kb fragments (each containing three predicted core promoter elements: P1, P2 and P3), and a 1.13 kb fragment (containing 2 predicted core promoter sequences: P1 and P2). The score values of P1, P2 and P3 were 0.88, 0.81 and 0.81 respectively.

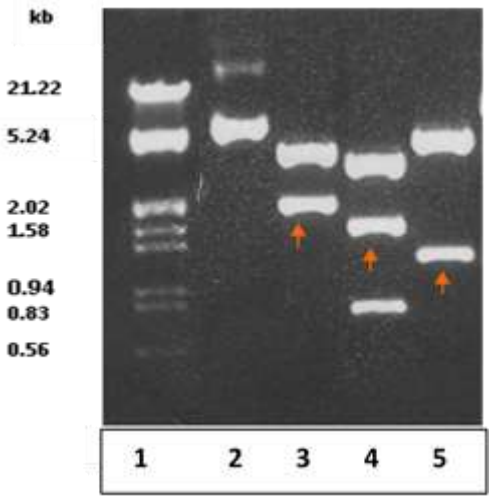

Figure S3 Restriction enzyme digests of the pGEM:2.2Promoter for the creation of constructs corresponding to 1.81, 1.51 and 1.13 kb *class-III chitinase* (*IF3*) promoter-containing fragments, respectively. Lane 1: Molecular Weight Marker III (Roche); Lane 2: uncut pGEM:2.2Promoter clone; Lane 3: *Nco*I/*BamH*I digest; Lane 4: *Nsi*I/*BamH*I digest; Lane 5: *Bgl*II/*BamH*I digest. The arrows indicate the expected 1.81, 1.51 and 1.13 kb *IF3* promoter-containing fragments.

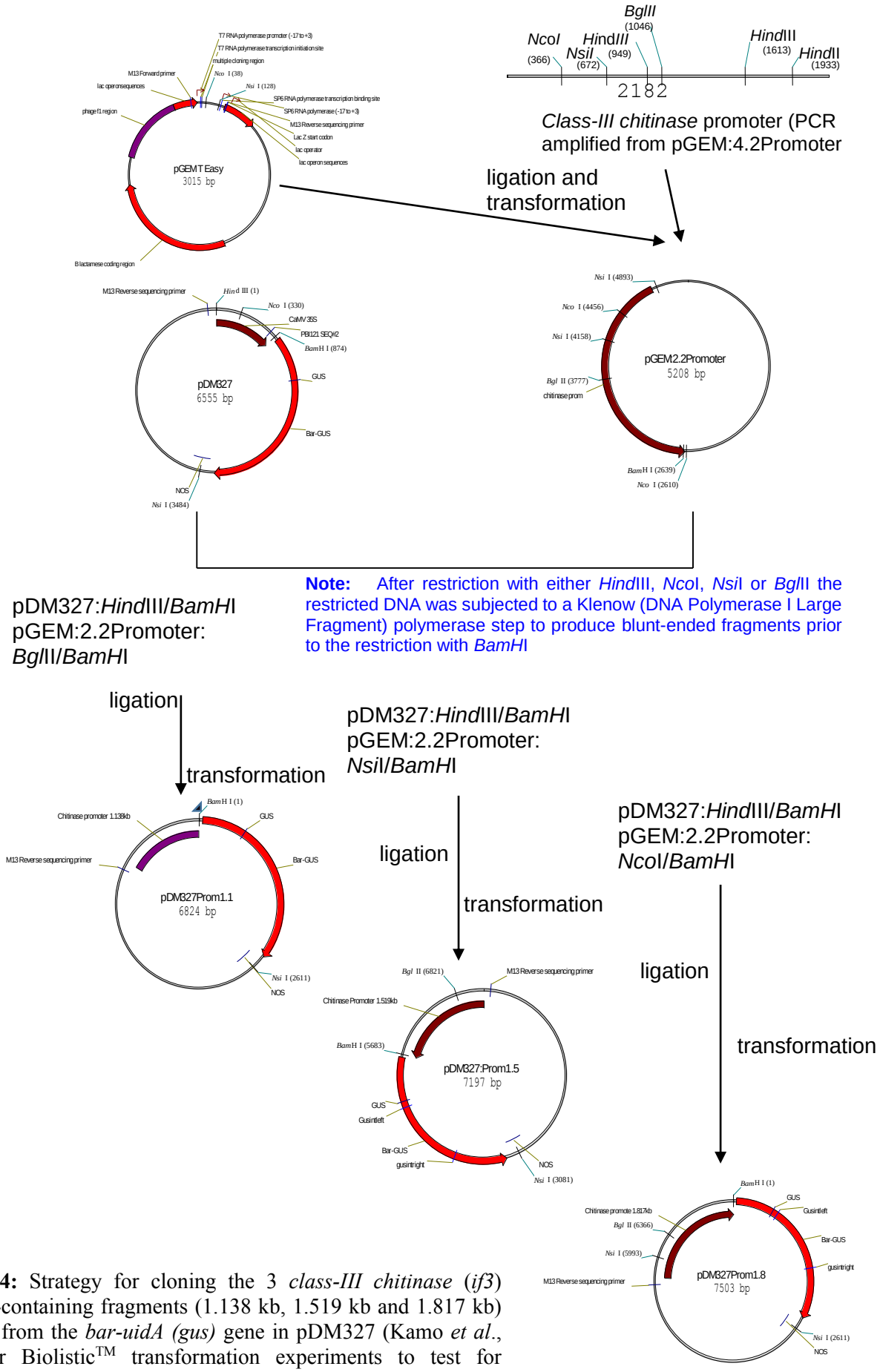

Figure S4: Strategy for cloning the 3 *class-III chitinase* (*if3*) promoter-containing fragments (1.138 kb, 1.519 kb and 1.817 kb) upstream from the *bar-uidA (gus)* gene in pDM327 (Kamo *et al*., 2000) for Biolistic™ transformation experiments to test for promoter activity in various plant tissues.