DOI: 10.1007/s10535-012-0281-1 BIOLOGIA PLANTARUM 57 (2): 341-350, 2013

Identification of promoter regions in the *Arabidopsis thaliana atExt1* extensin gene controlling late responses to wounding and pathogen attack

K. ROBERTS[1], G. MERKOUROPOULOS[1,2], and A.H. SHIRSAT[1]*

School of Biological Sciences, College of Natural Sciences, Bangor University, Memorial Building, Deiniol Road, Bangor, Gwynedd LL57 2UW, UK[1]
Institute of Agrobiotechnology, CERTH, Thermi, Thessaloniki, GR-57001, Greece[2]

Abstract

The *Arabidopsis thaliana* (L.) Heynh. *atExt1* extensin gene is expressed in a cell and tissue-specific manner, in response to developmental cues, and is inducible by a wide range of biotic and abiotic stresses. Over-expression of this gene has been shown to alter stem morphology and to limit the invasiveness of virulent bacterial pathogens, indicating that this cell wall protein gene plays an important role in plant development and defense. A detailed sequence analysis of 3.2 kb of the *atExt1* gene promoter region has identified a large number of putative 5'*cis*-acting elements. Based on the location of clusters of putative promoter control elements, seven *atExt1* 5' promoter truncations were constructed, fused upstream of the *β-glucuronidase* (*GUS*) reporter gene, and transformed into *A. thaliana*. Transgenic plants carrying the various promoter constructs were challenged by wounding and pathogen attack and analysed for *GUS* expression – this analysis revealed a complex pattern of regulation, involving positive and negative control regions. Northern analysis using wounded tissues from transgenic *Arabidopsis* plants carrying the 3.2 kb-promoter::*GUS* construct confirmed the transcriptional activation of the transgene.

Additional key words: β-glucuronidase, Northern blot, promoter truncations, transgenic plant.

Introduction

Despite being relatively abundant, the cell wall proteins of higher plants have not been the subject of intensive research, presumably because their precise role in the architecture of the plant cell wall remains somewhat elusive. Extensins are hydroxyproline-rich glycoproteins (HRGPs) containing the characteristic $Ser(Pro)_n$ repeat motif interspersed with tyrosine (Tyr) motifs. The occurrence of these tyrosine motifs is significant for the functionality of extensins, as it has been shown that cross-linking between Tyr residues on adjacent extensin monomers leads to protein insolubilisation (Brisson *et al*. 1994). Potential cross-linking and consequent insolubilisation of extensins within the cell wall has been proposed to result in wall reinforcement, thereby acting as a barrier to pathogen invasion as well as restricting further cell expansion (Cassab 1998, Lamport 2001). Recent findings involving electron and atomic force microscope observation of the *Arabidopsis rsh* mutant (a mutant that is defective in the *atExt3* extensin protein) showed that extensins form a positively charged scaffold which reacts with negatively charged pectins forming a complex which is important in cytokinesis. This indicates that extensins are also involved in the initiation of cell wall growth (Cannon *et al*. 2008).

There have been many studies exploring the developmental and stress-induced regulation of extensin gene expression (Showalter *et al*. 1985, Corbin *et al*. 1987, Memelink *et al*. 1993, Niebel *et al*. 1993, Tire *et al*. 1994, Shirsat *et al*. 1996, Hirsinger *et al*. 1999, Schenk *et al*. 2000, Petersen *et al*. 2000, Goodwin and Sutter 2009), but few studies to date have examined and identified promoter sequences controlling extensin gene expression. Guo *et al*. (1994) linked HRGP rice promoter truncations to *GUS* and reported a direct relationship between the length of the promoter and the activity of the GUS enzyme. Similar studies on the promoter of a maize

Received 27 July 2011, *accepted* 6 August 2012.
Abbreviations: GUS - β-glucuronidase; HRGPs - hydroxyproline-rich glycoproteins; PCR - polymerase chain reaction; Tyr - tyrosine.
Acknowledgements: We wish to thank Mr. Eleftherios Merkouropoulos for his valuable technical assistance in the preparation of the manuscript.
* Author for correspondence; fax (+44) 1248 370731, e-mail: a.h.shirsat@bangor.ac.uk

HRGP gene showed that intronic sequences located at the 3'untranslated region affected gene expression in a tissue- and a promoter-dependent manner (Menossi *et al*. 2003). Elliott and Shirsat (1998) identified *cis*-elements in the promoter of the *extA* extensin gene in *Brassica napus* that positively and negatively regulated responsiveness to wounding and tensile stress.

The *atExt1* gene is the best studied and characterised *Arabidopsis* extensin gene (Merkouropoulos 2000, Merkouropoulos *et al*. 1999, Merkouropoulos and Shirsat 2003, Roberts and Shirsat 2006, Wei and Shirsat 2006). This gene, which has been analysed extensively in the present study, has an unusual coding region in which $Ser(Pro)_4$ motifs alternate regularly with $Ser(Pro)_3$ motifs, reflecting an extraordinary molecular flexibility in the tertiary protein structure (Lamport 2001). Detailed analyses on the expression of the *atExt1* extensin gene have shown that this gene is developmentally induced, up-regulated during senescence and abscission, induced by wounding, infection with *Xanthomonas campestris*, and treatment with salicylic acid and jasmonic acid (Merkouropoulos *et al*. 1999, Merkouropoulos and Shirsat 2003). Roberts and Shirsat (2006) showed that increased levels of *atExt1* transcripts caused by over-expression of a *CaMV35S::atExt1* transgene in *A. thaliana* plants led to reduced inflorescence and stem elongation. Further work in the same system showed that *atExt1* overexpression dramatically reduced the infectivity of a virulent isolate of the bacterial pathogen *Pseudomonas syringae* (Wei and Shirsat 2006).

As the *atExt1* gene appears to be important in the plant response to wounding and pathogen attack, we were interested in identifying *cis*-acting promoter elements that controlled its expression. Analysis of 3.2 kb of the *atExt1* 5' flanking sequence identified an unusually large number of putative *cis*-elements Merkouroupoulos (2000). Based on this analysis, a series of *atExt1* promoter truncations were designed, inserted upstream of the *GUS* reporter gene, and transformed into *A. thaliana* plants. Transgenic plants were then analysed histo-chemically to identify regions of the promoter sequence controlling expression in response to wounding and pathogen attack. Northern analysis on wounded tissues from plants harbouring the 3.2 kb-promoter::*GUS* construct confirmed that the histochemical GUS staining observed was not an artefact.

Materials and methods

Plants and cultivation: *A. thaliana* (L.) Heynh. ecotype Wassilewskija (Ws-0, NASC line N1602) seeds, obtained from the Nottingham *Arabidopsis* Stock Centre (NASC), were sown in seed trays containing a 5:3 mixture of autoclaved *B&Q* multipurpose compost (*B&Q*, Chandlers Ford, Hampshire, UK) and *Vermiculite* (*William Sinclair Horticulture*, Lincoln, UK). Germination times were coordinated by stratifying seeds at 4 °C in the dark for three days. Stratified seeds were then transferred to a growth room with temperature of 20 - 25 °C, a 16-h photoperiod, and irradiance of 450 μmol m^{-2} s^{-1} provided by fluorescent lamps. The soil was kept moist by watering with distilled water and plants were fed every two weeks with full strength Hoagland's solution.

Construction of the *atExt1* promoter truncation::*GUS* fusion gene vectors: The sequence of the 3.2 kb *atExt1* promoter is shown in Fig. 1. The polymerase chain reaction (PCR) was used to generate *atExt1* promoter truncation fragments from the pGM2 plasmid template (Merkouropoulos *et al*. 1999), using primers designed to introduce artificial *Bam*HI and *Hind*III restriction sites (Table 1).

The forward primers Ext-110 to Ext-2209 are homologous to sections of the *atExt1* gene promoter and were used to generate the 5'-ends of the promoter truncations, up to and including the nucleotide stated in the primer name. In addition, the Ext-110 to Ext-2209 primers were all designed with a synthetic *Hind*III site at their 5'-ends to facilitate cloning of the amplified product into the pBI101 *GUS* expression vector. The reverse primer Ext-R was designed to generate the 3' end of the promoter fragment and to introduce a synthetic *Bam*HI site (Fig. 2*A*). This primer is homologous to a section of

Table 1. Primers used to generate the *atExt1* promoter truncation fragments from the pGM2 plasmid template. Restriction sites recognised by the *Bam*HI (*in italics*) and *Hind*III (*in bold*) restriction endonucleases have been introduced.

Primer name	Primer sequence
Ext-R	5'-GTCGCA*GGATCC*ATTGGTGCCCCCATTGTG-3'
Ext -110	5'-GCAGTG**AAGCTT**CCATGAGAGATAGTCGTAAAAAG-3'
Ext -412	5'-GCATGT**AAGCTT**GTTTGGTTAACATATTTCATAC-3'
Ext -883	5'-GTCGCT**AAGCTT**GACCTTAACGCGTTACTTCTTT-3'
Ext -1226	5'-GCAGTG**AAGCTT**CCTTTATCGTTGAGTTGTTTAC-3'
Ext -1747	5'-GCATGT**AAGCTT**GATATCAGATCGTATTTTTCTC-3'
Ext -1963	5'-GCAGTG**AAGCTT**CCTTTTGTTATCCTTTTTGGT-3'
Ext -2209	5'-GTCGCT**AAGCTT**GTGTTCTTTCTTTTCCATTCATT-3'

```
-3200 AAAATGAACA TCAAGAATAC TACAAAACAA CCATTAATAA AAAATTGTTC AGGTGAATTT TCCGGTAATG GAGGGAGCCT
-3120 TTCCATCACC AGCTTTAACG ATTCTAAGTT TCTAACCCAA TTTTTTATGA GAAATCAATC AGAATCAGCC ATGTTGGAGG
-3040 AAAAAAGACT CTTTACTTCT TTAACTTTTG CATGGAATTT TGCTTTAAAG TCATTGATTT ATTTGGTAGA TCATTATGAA
-2960 AATACAAGCT CGTATTTTGT TAATATAAAA ATGAGTGTGG AGGAGAAGAA ATCAATACAC AAATTGTTGA CCATCATTTT
-2880 TAGGTAAATA GTATAATTGT TTTTCTTTTT TCTTTTTCTA AATAGTATAA TTTTGTTTAT ATGAAAAATG ATTAGTTTCT
-2800 AATAATTTAC GTTTTATGAA CAAACGAGAA CCGTGTCCGT AATTCAGTAC GCGGGGGATC ATTAAGACAA GTTGTAGCGA
-2720 ATAGTAAGTA CTAAGTAGTG ATGTTTGCTG AGATATACAC ATAAAACAGA AAGTAAAACT TAGAACATGT CAGTAACATA
-2640 CCATATATTT CATTATTGCT TTAGCACATT GACACATTCA TATTATTATT ATTCTTTGGT ATGAACATTC ACATTACTTA
-2560 TGTTATGTTA TAGTCTCAAA ATTAATTAGG AATGTAAGGA AAAAACAAGG CATCACTTGG AAAAGAAAAA TATAAATGAG
-2480 AATAGTAATT AAAAAAAATA ATTATTATTT TTCTTTTTGT CGAGGGTACT TTAAAATTCC ATATGTATAA ATATAATATA
-2400 CTCTAAGAGT ATCAAAATAA AGTTGAATCA CTAAATCCAA TAGTTGGCGA TTTATTGGGA CAAATTTTTA TTCATTTATT
-2320 GAAAATTTGA AACACTGGGC AAAGTGTGCA ATACGACAAA ATAAACTCCA TAGGTGTTTA CCAGGGAATA GTAACAATTA
-2240 AGATGGTTCG ATTATATGTG TGTTCTTTCT TTTCCATTCA TTTGAATTTG GGTGTATATA TATGATAATA TTATTATTAT
-2160 TTATAAAAAT AAATCAACAA CACGTAGCTA ATAGTTAGTT GATATGAATA TATTATCTCC GAAGAGAAGA TAATAAATCA
-2080 ACCAGCAGCT TTGTTGACAA AAATAAAAAT AAGAATATGG AGCTTAGAAA TTACATATTA TCATTTATCA ATCAAATAGC
-2000 TTCTTTGTTT TTTTACTTTT TTTTTCCTTT TGTTATCCTT TTTGGTACTT GGCTTGGACT TTGAAGTACC AAACGATGTT
-1920 TCGCATTTGC CTAATTTTCC TAAAAACACT AGTAACGTTT TTGAATAAAA CAAATTTGAA CTCAAAACTT CAAAAGGCAA
-1840 CCGTATTCTT GAATTATGTT TCTGTTTAAT GTTTGTTATA ATCACTACTT ATTAACTGAA CTTCAAAAGG CAACCATATT
-1760 TGATATCAGA TCGTATTTTT CTCATGCTTT CCGAACAAAT ATTTTGTATT CAAACATTAG TATTGTTTCT AAATAACTCA
-1680 AACCTAATTA AATTTACCTG ATCTAAATTA TTAATGCCGG TGATTATAGT AATTTTGTTT GAAGTCGATT TCTTCTATAT
-1600 CAAAATTGTC AAGATTAGTT ATAGAACTTT TGACGCCTAA CCAAAATAGT CAAATCTCGA GAAAAAATAC TATATAGAAT
-1520 GTTAAGTTGT TGATTTGTCT ATATCATAAT CCAGCTGGTT TACTTACCCA TTTGATAAAA ATCAAAAGAA AAAATCCCAT
-1440 TTAATTTTAT TATCAAAGAT GTTATATTGG CTTATGTTGA CCAAGTTTCG ATATTATAGT TTATTGACCT ATTTTCGATC
-1360 TTAGCCTTAA ATTGGATTAT CCTCCTCCTT ATACATTTCT AAAACACCTG TTCGTGATAA GATAATTCCC AATCTGTTAA
-1280 CATTATATGA AAAACAATAT AAATATTGTC AATATGTTAT GGCCTTTATC GTTGAGTTGT TTACATCATT CACAAGCAAG
-1200 CAGCTAAAAC AAAATTCACC ACAAGTTACA ATATGTTGTC TTGATCGTAA AATGTATTTA GGTTTGAATT AATGTTAGAC
-1120 TAATTAATAA TTAGATAAAT TTAAGAGTTG CGAGGGATTA ATAAGCGTAA TGACAAAAGA AACGTGTAAG AGTAATAGAA
-1040 AACATATGAA TTAAAAACCT AAAAACAAAA GTAAGAAGTT AATCAAAATT GACTGTCACC GGCACGAAAG AGAATCTAGT
 -960 ACATGACGGA CATCTTTCTA CTTCAATGTT CCTTCTATAG TCAACTAGGC CGCCTATATC TTCAAGACCT TAACGCGTTA
 -880 CTTCTTTATT TATTTTTTAT TTTTTTGCTT AGAATTCGTA TTTCGTTTTG CGATGAACAT GTAAATCATA AGATAATTTG
 -800 GAATATTAGT TTTCATTCGC CACTAAATCT TTCAATACAT TATTTTGGGT GATGAAAGCA CAACTTGGCA TTGAAAGTTT
 -720 GAAACACATA ATAGGTTTAA GAAATTTAGT GGGAAGCTAA ATATCCATGA GATTATTTTG TGGATTGTGC AAACATTGTA
 -640 GAGACATAAG TTATTATCGG ATTGCAGACG TTGCAAAAAT GTGTACACAA CTGTTAACAT ATGATCATAT CATACCATAT
 -560 GGCCTCGTTT CTAGAATTTC ATGTGCTGTT AAATGAAGTA ACTGTTAACA TTTACCATAC TCAGAAATGG ATGTATTATC
 -480 CTCTCATATG ATCTTTCTTC AGGCCGATCT ACCTCTCGAT CAATTCTTGT AGTTTTGTTT GGTTAACATA TTTCATACTT
 -400 AAATTCTGTT AGCTTTAGAT TTTTGTTTTC TTCTTCTTCT TTTTTGGTAT AATCCTGTTA TCTTTAGATT CAATGTTCTC
 -320 TATATTATAT GTGATTTTAT ACGTTCGGAA CATTCAGAAC ATGTCGATCA ACTACGACCG AAAATAATAG AAGGGAAGAA
 -240 GAGAATCAAT AATGAAACCC GAGCGAGGGA GACTAACCTC ATTTTCTTTC TTTGTATTAT TAATATAGTC AATGTCTTTT
 -160 TTTGTATTGC TTACACACAA CAGGACACAA ATTTACTACC ATGAGAGATA GTCGTAAAAA GGGATAAAAT GTAAATGTGA
  -80 GAAGCTATAT AAAGGATGCT AACGGGAGAG AGTAAGAGCA TCAAACACAA AGCAAAACAC ATAACACAAT GGGGGCACCA
   +1 ATG
```

Fig. 1. Sequence of the 3.2 kb *Arabidopsis* extensin *atExt1* promoter.

the *atExt1* coding sequence which includes a second ATG, thus incorporating both ATG codons in the amplification product. The presence of this *atExt1* coding sequence in the *atExt1::GUS* fusion gene resulted in the addition of thirteen amino acids to the N-terminus of *GUS* (Fig. 2*B*). As it has been reported that the *GUS* reporter gene tolerates additions to its N-terminus without a noticeable effect on activity, adverse effects of this N terminal addition were not expected (Gallagher 1992).

PCRs were carried out using the high fidelity enzyme *Tfu* DNA polymerase (*Helena BioSciences*, Sunderland, UK) in order to minimise the error rate of replication. Purified and amplified DNA was then digested with *Bam*HI and *Hind*III, electrophoresed on a gel and the digested DNA bands extracted and purified. The *Hind*III-*Bam*HI promoter fragments were then ligated into the *pBI101.2* vector (cut with *Hind*III and *Bam*HI and then dephosphorylated) in a cohesive-end ligation reaction using a vector: insert ratio of 1:3. Ligation products were electroporated into *Escherichia coli* DH5α cells and screened on selective Lurie-Brot (LB) media plates (35 μg cm^{-3} kanamycin). Colonies that grew were then analysed for the presence of the recombinant binary vectors using colony-PCR. Positive colonies were further analysed by restriction digests of plasmid DNA with *Hind*III and *Sac*I to excise the promoter fragment and the *GUS* coding sequence. Sequencing was used to verify that the plasmid border sequences remained intact. Plasmids, and subsequent transgenic plants, were named according to the size and location of the promoter truncation, *i.e*. -110, -412, -883, -1226, -1747, -1963, and -2209.

***A. thaliana* transformation and characterisation of the transgenic plants:** The *Agrobacterium tumefaciens* strain LBA4404 was transformed with the new plasmid constructs by tri-parental mating, using the helper plasmid pRK2013. Successfully transformed *A. tumefaciens* strains were selected on the basis of kanamycin resistance. *Arabidopsis* plants were transformed with the *atExt1* promoter truncation::*GUS* constructs using the simple floral-dip method (Clough and Bent 1998). Successfully transformed seed lines were selected on the basis of kanamycin resistance. Characterisation of transgenic plants by DNA analysis was performed on tissue from T_2 plants (T_0 plants were those

initially treated). Individual transgenic lines were analysed for the presence and integrity of the *atExt1* promoter truncation::*GUS* transgene by Southern hybridisation, and all subsequent analyses were performed on T_2 plants and their progeny (data not shown).

X-Gluc treatment of *GUS*-expressing *A. thaliana* transgenics: Expression of the *GUS* gene in transgenic plants was visualised by the method described by (Jefferson *et al*. 1987). Samples were incubated in 50 mM NaH_2PO_4 (pH 7.0), containing 1 mM 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) dissolved in dimethyl formamide and 18 mM cycloheximide at 37 °C overnight. Samples were then fixed in 3 % (v/v) glutaraldehyde at 4 °C overnight, then dehydrated in successive washes of 25, 50, 75, and 100 % denatured ethanol each for at least 1 h, and finally cleared and stored in 100 % denatured ethanol at 4 °C. Synthesis of the GUS protein was also investigated in wild-type plants not carrying the transgene, and in these cases GUS staining was never observed. Samples were photographed on a *Leica Wild M8* microscope, using *Agfa Ultra 50* colour film, or with a *Canon EOS 300D* digital camera.

Wounding and pathogen infection of transgenic *A. thaliana* leaves: Individual *Arabidopsis* leaves from plants containing the *atExt1* promoter truncation::*GUS* fusions were wounded by puncturing the leaf surface several times with a metal pin (Savenstrand *et al*. 2002). The pin was sterilised after wounding each leaf by immersion in ethanol and air-dried. Leaves were excised and analysed for *GUS* expression 0, 24, 48 and 72 h after wounding. For Northern analysis, leaves were either sliced into 3 - 4 mm strips and incubated on filter paper moistened with phosphate buffer for 12 and 24 h, or folded along the leaf lamina and incubated as before for 48 h prior to RNA extraction. Regardless of the wounding technique used, there was always expression of the *atExt1*::GUS fusion around the wound site (Merkouropoulos and Shirsat 2003).

The *Arabidopsis* pathogen *Xanthomonas campestris* pv. *campestris* strain 8004 *(Xcc* 8004) was provided by the John Innes Centre (Colney, Norwich, U.K.). Prior to use, freshly grown cultures were pelleted by centrifugation at 2 500 *g* for 5 min, and washed twice in 10 mM $MgCl_2$. Bacterial cells were then resuspended in distilled H_2O to give a final concentration of 10^6 cfu cm^{-3}. *Arabidopsis* leaves were infected with *Xcc* 8004 by pipetting 2 mm^3 of the 10^6 cfu cm^{-3} sample on the abaxial side of the leaf lamina. The infected plants were then covered with clear plastic lids until individual lesions were examined. Five days after infection, lesions that developed on leaves from the transgenic plants carrying the *atExt1* promoter truncation::*GUS* gene constructs were examined for *GUS* expression. Control plants were treated with 2 mm^3 distilled H_2O. For Northern analysis, leaves which showed visible signs of infection after 5 d were used for total RNA extraction.

Northern hybridisation: For Northern analysis, total RNA was isolated from *Arabidopsis* leaf tissues using a hot phenol protocol (Kay *et al*. 1987). Total RNA (10 μg) was separated on a 1.3 % formaldehyde-agarose gel and transferred by capillary blotting to a *Hybond*^{N} nylon membrane as described by Sambrook *et al*. (1989). The *Arabidopsis atExt1* coding sequence (Merkouropoulos *et al*. 1999), was radio-labelled with [^{32}P]dCTP by random primer labelling and used as a probe in northern hybridisations as described previously (Elliott and Shirsat 1998). Blots were washed to a high stringency with the final wash being at 65 °C in 0.1× SSC with 0.1 % SDS to remove background and non-specific hybridisation signals. Post hybridisation membranes were exposed to autoradiography films for varying lengths of time.

In-silico promoter analysis: A 3.2 kb region of the *atExt1* extensin gene promoter (Fig. 1) was inspected using *MatInspector* for possible matches to the sequences included in the *TRANSFAC* database releases 2.1 and 2.2, with the core similarity set to 0.80 or 0.85 (80 or 85 % identical to the core consensus, respectively) and the threshold to the matrix similarity set to ≥ 0.8. Due to the limited number of plant transcription factor binding site entries in the database an additional compilation of transcription factor binding sites collected from the literature was also used. This second database was constructed by Elliott (1998), with particular attention drawn to elements shown to be important for the developmental, organ-specific expression of plant genes and expression in response to wounding and infection.

Results

Analysis of the *atExt1* promoter sequence by Merkouropoulos (2000) allowed the design of seven promoter truncations, the size and range of which were based on the identification and location of putative *cis*-elements in the 5' flanking promoter region. Elements given the most attention were those considered to be responsible for induction by stress. The promoter truncations were PCR amplified and cloned – the PCR strategy used positioned the 5' forward primers so as to avoid regions of the promoter sequence containing a high concentration of putative control elements, thus limiting the disruption of individual control motifs. It is notable that putative W-box motifs appear frequently in the sequence, with the TGAC core motif occurring at seventeen positions in the 3.2 kb sequence, eleven of which are present within a 677 bp section located between -904 to -1581 bp.

These promoter truncations were inserted in the sense orientation upstream of the *GUS* coding sequence in the pBI101.2 expression vector. Plasmids were named pKB7

(plasmid-110; pBI101.2 vector plus 110 bp *atExt1* promoter fragment) to pKB13 (plasmid-2209; pBI101.2 vector plus 2209 bp *atExt1* promoter fragment). A plasmid map of the new constructs is shown in Fig. 2*A*, with the *atExt1* promoter-pBI101.2 border sequences detailed in Fig. 2*B*. The promoter truncation constructs and the original pGM2 construct containing 3.2 kb of the *atExt1* promoter sequence, were transformed into wild-type *Arabidopsis* plants in order to examine their expression in a homologous system. Successful transformants were selected on the basis of kanamycin-resistance and further selected by Southern analysis. Any plant lines showing evidence of recombination in the transgene were discarded (data not shown).

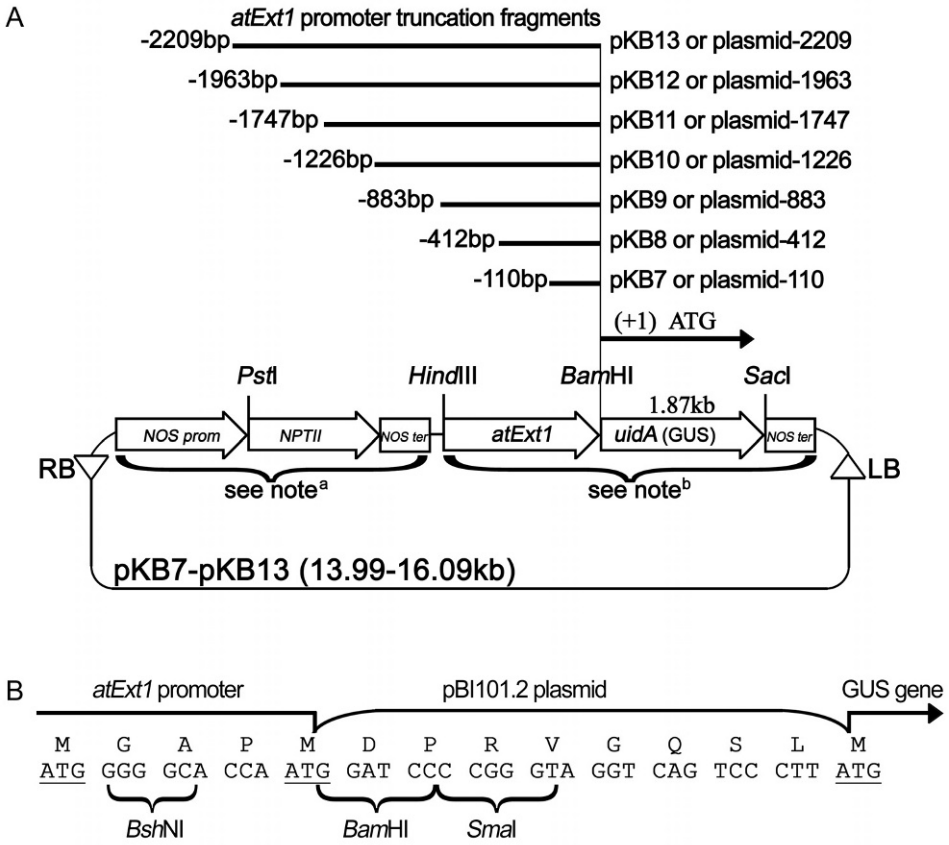

Fig. 2. *A* - Map of the recombinant plasmids pKB7 to pKB13, showing major restriction sites and features. The *arrows* indicate the 5' to 3' orientation; note[a] - the chimeric kanamycin resistance gene: *nopaline synthase* promoter (*NOS* prom)::*neomycin phosphotransferase* (*NPT II*)::*nopaline synthase* terminator (*NOS* ter); note[b] - The *atExt1*::*GUS* transgene *atExt1* promoter truncation (*atExt1*)::reporter gene *uidA* (*GUS*)::*nopaline synthase* terminator (*NOS* ter). *B* - Fusion junction of the inserted *atExt1* promoter truncations linked to the *GUS* reporter gene showing the reformed *Bam*HI restriction site and the position of the *atExt1*-derived start codons in relation to the ATG of the *uidA* (*GUS*) gene. The ATG start codons are underlined. The translated sequence is shown above the nucleotide sequence. Major restriction sites are also shown.

Plants were wounded by inserting a sterilised pin into the lamina of the leaves and analysed for *GUS* expression at 0, 24, 48 and 72 h following wounding (Fig. 3*A*). Expression at intermediate time points (6 and 12 h) was not seen. Synthesis of GUS at the wound site was not seen at any time point following wounding in transgenic plants carrying the -110, -412, or -883 bp promoter truncations. Localised faint expression around the wound site was first seen in plants carrying the -1226 bp construct, but only in leaves analysed 72 h after wounding. This expression pattern was maintained in plants carrying the -1747 bp construct. Surprisingly, plants carrying the -1963 bp construct showed no wound induced expression at any time period. Expression of *GUS* around the wound site was restored in plants carrying the -2209 and -3200 bp constructs at the 24, 48 and 72 h time points, suggesting that the -883 to -1226 bp promoter region contains elements which activate a late wound response while the -1747 to -1963 bp region contains elements that repress this activation. The promoter region between -1963 to -2209 bp must also therefore contain elements which are able to overcome the repression exerted by the -1747 to -1963 bp region, as these plants show a relatively strong expression of *GUS* around the wound site 24 h after the onset of the wounding stimulus. GUS staining was stronger around the wound site on leaves carrying the -2209 bp and the -3200 bp constructs, suggesting the presence of a sequence motif directing expression at this region. Northern analysis using leaf tissues from transgenic

Arabidopsis plants carrying the -3200 bp construct which had been wounded showed high levels of transcriptional activation of the *atExt1* gene 24 h after leaf slicing, and 48 h after folding the leaves but not earlier (Fig. 3*B*), confirming the observed GUS staining.

The transgenics carrying the -110, -412, and -883 bp promoter truncations showed no expression in response to infection with *Xcc* 8004 (Fig. 4). Faint *GUS* expression around the edges of the lesion was first seen in transgenics carrying the -1226 bp truncation and expression became more intense in the -1747 bp construct. Expression in plants carrying the next longest promoter truncation (-1963 bp) was not seen. Expression around the edges of the lesion was restored in transgenics carrying the -2209 and -3200 bp constructs. This pattern of expression is similar to that seen in the wounding

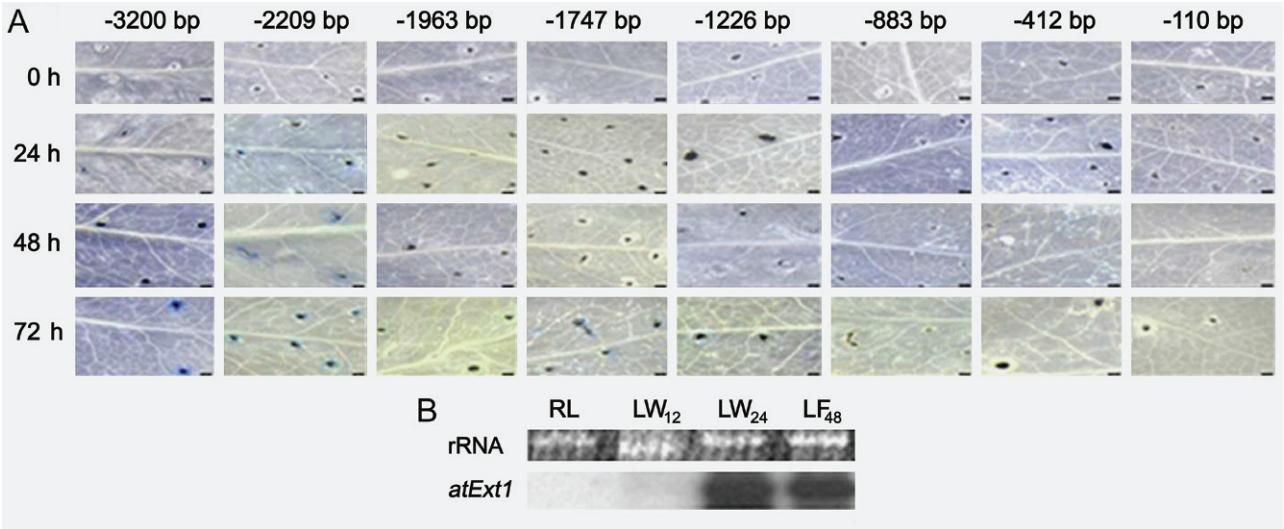

Fig. 3. *A* - Expression of the transgene in response to wounding. Expression of *GUS* in transgenic leaves carrying the *atExt1* promoter::*GUS* gene fusions 0, 24, 48 and 72 h after wounding the leaves with a sterilised pin. The length of the promoter truncations is indicated at the top, while the time is indicated at the left. Images shown are representative of 15 leaves from 7 clonal plants. The experiment was repeated twice. *Bar* = 1 mm. *B* - Expression of the *atExt1* gene in transgenic *Arabidopsis* plants harbouring the 3.2 kb promoter::*GUS* fusion construct. Samples collected from rosette leaves (RL), leaf strips 12 and 24 h after incubation in phosphate buffer (LW_{12}, LW_{24}), and folded leaves 48 h after incubation in phosphate buffer (LF_{48}). Total RNA was extracted and 10 μg of each sample were run on an agarose gel where the equivalence of RNA loading between tracks was checked by ribosomal RNA (rRNA) bands intensity. The gel was Northern-hybridised against the *atExt1* gene coding sequence and an autoradiograph was produced after exposure of the blot against an X-ray sensitive film.

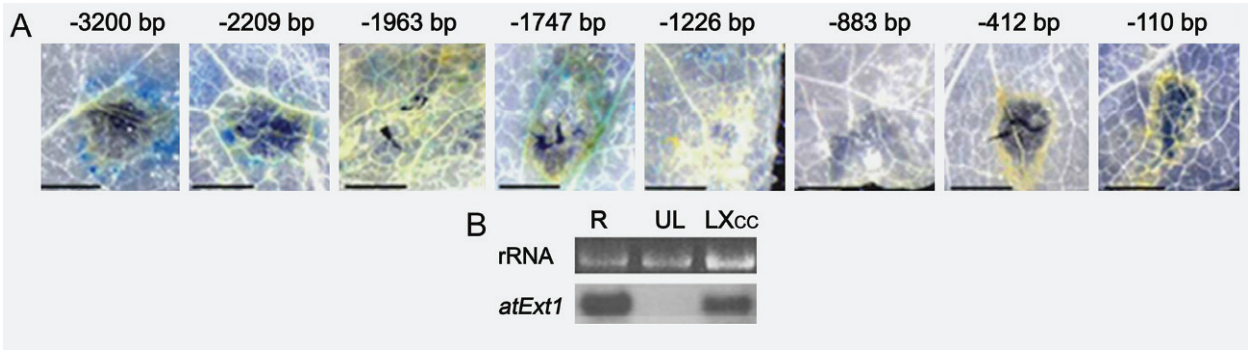

Fig. 4. *A* - Expression of the transgene in response to pathogens. Expression of *GUS* in lesions produced on transgenic plants containing *atExt1* gene promoter truncations in response to infection by *Xanthomonas campestris* pv *campestris* 8004 (samples were taken 5 d after the infection). The length of the promoter truncations is indicated at the top. Images shown are representative of 15 leaves from 7 clonal plants. The experiment was repeated twice. *Bar* = 1 mm. *B* - Expression of the *atExt1* gene in transgenic *Arabidopsis* plants harboring the 3.2 kb promoter::*GUS* fusion construct. Samples collected from roots (R), uninfected leaf (UL) and leaves infected with *Xcc* 8004 (LXcc). Total RNA was extracted and 10 μg of each sample were run on an agarose gel where the equivalence of RNA loading between tracks was checked by ribosomal RNA (rRNA) bands intensity. The gel was Northern-hybridised against the *atExt1* gene coding sequence and an autoradiograph was produced after exposure of the blot against an X-ray sensitive film.

Table 2. Major putative *cis*-acting elements contained 8 different promoter regions which have been analysed with respect to *GUS* expression. The motifs mentioned in the table are referenced according to numbers in superscript: 1 - Yamada *et al*. 1994, 2 - Meshi and Iwabuchi 1995, 3 - Dröge-Laser *et al*. 1997, 4 - Klotz and Lagrimini 1996, 5 - Petersen *et al*. 2000, 6 - Lebel *et al*. 1998, 7 - Goldsbrough *et al*. 1993, 8 - Yu *et al*. 2001, 9 - Pastuglia *et al*. 1997, 10 - Yamada *et al*. 1992, 11 - Mason *et al*. 1993, 12 - Rouster *et al*. 1997, 13 - Ohme-Takagi and Shinshi 1990, 14 - Sessa *et al*. 1993, 15 - Elliott and Shirsat 1998, 16 - Hauffe *et al*. 1993, 17 - Hamilton *et al*. 1998, 18 - Wingate and Ryan 1991, 19 - Kawaoka *et al*. 1992, 20 - Eulgem *et al*. 1999, 21 - Solano *et al*. 1995, 22 - Izawa *et al*. 1993, 23 - Boyle and Brisson 2001, 24 - Sasaki *et al*. 2006. Numbers in subscript show the frequency at which the motifs are found in the particular promoter site.

Promoter section [bp]	Putative element	Site name
+1 to -110	core promoter element	TATA box
	pathogen responsiveness	Elic-I[1]
-111 to -412	general stress responsiveness	ACE [2], H-box[3]$_2$, TC-rich repeat [4]$_2$
	pathogen responsiveness	Elic-I[1]$_2$, PR-PRE [5], TTGACT[6]$_2$
	induction by salicylic acid	TCA motif[7]$_6$, TTGAC[8]$_2$,
	wound responsiveness	PR-PRE[5], TTGACT[6]$_2$
-413 to -883	general stress responsiveness	ACE[2], H-box[3]
	pathogen responsiveness	Elic-I[1]$_8$, bZIP[2], WUN[9]
	wound responsiveness	WUN[9]
-884 to -1226	general stress responsiveness	ACE[2], bZIP[2], Pal Box-IV[10]
	induction by jasmonic acid	MeJa Box 1[11], TGACG[12]
	pathogen responsiveness	Elic-I[1]$_5$, PR-Box (GCC box)[13], TGACG[12], TTGACT[6]$_2$, WUN[9]
	expression enhancer	QAR[15], QuantElem[16]
	induction by salicylic acid	TGACG[12], TTGAC[8]$_2$
	wound responsiveness	Athb-1[14], PR-Box (GCC box)[13], TTGACT[6]$_2$, WUN[9]
	wound repression	SEBF[23]
-1227 to -1747	induction by jasmonic acid	TGACG[12]
	pathogen responsiveness	Elic-I[1]$_7$, TGACG[12], TTGACC[6]$_2$, TTGACT[6], TTTGACT[20]
	expression enhancer	Q element[17]
	induction by salicylic acid	TTGAC[8]$_6$, TGACG[12]
	wound responsiveness	Athb-1[14], Pin I[18], TTGACT[6], WUN[9]$_3$
-1748 to -1963	general stress responsiveness	ACE[2]
	expression enhancer	enhancer (Enh)[19]
-1964 to -2209	general stress responsiveness	ACE[2], bZip[2]$_2$, MYB.Ph3[21]
	pathogen responsiveness	Elic-I[1]$_5$, WUN[9]$_2$
	expression enhancer	G-box[22]
	induction by salicylic acid.	TTGAC[8]
	wound responsiveness	-624 box, G-box[22], WUN[9]$_2$
-2210 to -3200	general stress responsiveness	ACE[2], Pal Box-IV[10], TC-rich repeat[4]
	pathogen responsiveness	Elic-I[1]$_3$, WUN[9]$_2$, TTGACC[6]
	induction by salicylic acid	TTGAC[8]
	wound responsiveness	Athb-1[14]$_3$, WUN[9]$_2$, VWRE[24]

Table 3. Location of 17 copies of the W-box core sequence (TGAC) within 8 different promoter regions which have been analysed with respect to *GUS* expression. Locations in bold indicate the 5'-3' orientation of the core motif, whereas the remaining locations are in 3'-5' orientation.

Promoter section [bp]	Location of the W-box core sequences
+1 to -110	
-111 to -412	-173
-413 to -883	
-884 to -1226	-921, **-957**, -986, **-991, -1070**
-1227 to -1747	-1253, **-1376**, **-1403**, -1552, **-1570,-1593**
-1748 to -1963	
-1964 to -2209	-2206
-2210 to -3200	**-2611**, -2652, **-2893, -2991**

experiments and indicates that the *atExt1* gene is activated in response to pathogen infection by promoter elements located between -1226 to -1747 bp. In common with the wound response, elements located between -1747 to -1963 bp act as repressors with the repression being overcome in transgenics carrying the -2209 and -3200 bp promoter constructs. Northern analysis in transgenics carrying the -3200 bp construct showed strong expression of *atExt1* mRNA in leaves which had been infected with *Xcc* 8004 confirming that the observed GUS staining in the leaves carrying the -3200 bp construct was not an artefact (Fig. 4*B*). As a positive control, Northern analysis showed a high expression in uninfected roots – this is the normal pattern of expression in wild type *Arabidopsis*.

Discussion

Induction of the *atExt1* gene promoter in response to wounding: Previous work on *atExt1* gene expression (Merkouropoulos *et al*. 1999) showed that wounding of either the stem or leaves resulted in an increase in extensin mRNA that was detectable at 12 h following treatment, reaching a maximum at 24 h and declining at 48 h. This time-scale correlates well with the visible GUS staining seen in transgenic plants carrying the -2209 and -3200 bp *atExt1* promoter truncations, where expression was first seen within 24 h after wounding, and continued to be observed to the 72 h time point. The GUS staining patterns observed show that the control of wound-induced gene expression by the *atExt1* gene promoter is complex. Three regions of the *atExt1* gene promoter appear to be involved in the wound response. The -1226 to -1747 bp promoter region directs activation at the wound site 72 h after wounding; this expression is suppressed by elements contained within the -1747 to -1963 bp promoter region whereas elements located between -1963 to -2209 bp overcome this repression and induce GUS synthesis in response to wounding at the 24, 48 and 72 h time points.

Induction of the *atExt1* gene promoter by infection with *Xcc* 8004: Research on a number of host-pathogen systems has shown that extensin content rise in response to pathogen ingress and proliferation (Cassab 1998, Humphrey *et al*. 2007). The bacterial pathogen *Xcc* 8004 has been shown to establish a compatible interaction with *A. thaliana* and Merkouropoulos and Shirsat (2003) demonstrated that infection with *Xcc* 8004 activated the *atExt1* promoter in transgenic *A. thaliana* plants carrying the -3200 bp *atExt1* promoter::*GUS* fusion gene. In the present work, the study of the *atExt1* response to bacterial infection was taken further and showed a localised response around the mature lesion in late stages of infection indicating that the promoter control element(s) involved in this pathogen response were located between -883 to -1226 bp. This response is similar to that seen in virus-infected *N. tabacum* leaves carrying the *Phaseolus vulgaris HRGP1.4* promoter::*GUS* transgene where GUS staining was restricted to a ring of tissue surrounding the developing lesion (Wycoff *et al.* 1995), and also in transgenic *Arabidopsis* plants expressing a full length *atExt1*::*GUS* fusion infected with *P. syringae* DC3000 (Wei and Shirsat 2006).

Negative regulation of wound and pathogen induced expression of the *atExt1*::*GUS* fusion gene between -1747 and -1963bp in the *atExt1* promoter: Expression of *GUS* was observed in plants carrying the -1226 and -1747 bp promoter truncations but not in plants carrying the -1963 bp truncation, both in response to wounding and pathogen attack, suggesting repression of both responses by a control element(s) located between -1747 to -1963 bp upstream of the translation start site. A search of this region for wound and pathogen response regulatory elements did not reveal homology to any known negative regulators of the wound and pathogen response, however the -883 to -1226 bp region contains two motifs which have been shown to act as negative regulators in other systems; a putative SebF motif located at -976 bp, and a GCC box like sequence located at -900 bp. The SebF motif was firstly described in the *Solanum tuberosum* pathogenesis-related *PR-10a* gene promoter where it was found to be a binding site for the silencing element binding factor SEBF, which led to the transcriptional repression of the *PR-10a* gene (Boyle and Brisson 2001). The GCC box like sequence (AACTA GCCGCC) resembles the sequence TAAGAGCCGCC, which was found to negatively regulate the wound induced *ERF3* gene in tobacco (Nishiuchi *et al*. 2004). It is possible that these two sequences, the SebF motif and the GCC-box like sequence, function in combination with yet unidentified elements located between -1747 to -1963 bp in the *atExt1* promoter to repress wound and pathogen induced expression, though more experimental analysis is needed to confirm this possibility. This repression is overcome by elements located in the -1963 to -3200 bp promoter region. This region contains a number of elements that have been shown to regulate wound responsiveness as well as responses to general stresses (Table 2).

While wounding and necrosis due to pathogen attack might superficially be considered to be the same stress, it is evident that *Arabidopsis* responds to these stresses differently with respect to *atExt1* expression. The expression time points are different. In the biotic stress occasioned by *Xcc* 8004 infection, promoter elements between -883 to -1226 regulate expression, whereas the expression pattern in response to mechanical wounding is complex, being regulated by positive and negative control elements located in different regions of the promoter. Cheong *et al*. (2002) using microarray analysis showed that *Arabidopsis* genes which were activated by wounding were also activated by pathogen attack indicating the integration of the wounding and pathogen response. While the conventional view is that different pathways regulate the plant response to biotic and abiotic stresses (the wound response being regulated through the octadecanoid pathway and the pathogen response through the synthesis of salicylic acid *via* the phenylpropanoid pathway) it is becoming increasingly apparent that these pathways are not functionally distinct, and indeed engage in cross talk. The advantage of responding differently in respect of *atExt1* promoter activation to pathogen infection and mechanical stresses, is however, unclear, as the end effect is the putative strengthening of the cell wall by an increase of extensin deposition.

Clustered putative W-boxes in the *atExt1* gene promoter: In addition to putative *cis*-elements that confer gene inducibility by various wound-signalling compounds, there are many more putative motifs within the *atExt1* gene promoter sequence that may control

wound and pathogen induced activation. Potentially the most interesting of these elements are the W-box motifs (Eulgem *et al*. 2000). W-boxes occur in the promoters of numerous plant defense genes, and are frequently clustered together (Lebel *et al*. 1998, Rushton and Somssich 1998) and can function synergistically (Eulgem *et al*. 1999). When arranged as multimers, W-box sequences are sufficient to respond to a range of pathogens, elicitors and wounding (Rushton *et al*. 2002). In the 3.2 kb *atExt1* gene promoter sequence, the TGAC core motif of the W-box occurs 17 times, with 11 of these motifs clustered within a 677 bp section between -1581 and -904 bp upstream of the translation start site (Table 3). This promoter region is contained within sections of the -1226 and -1747 bp promoter truncations and it is possible that the W box elements in this region in combination with unknown elements in the -883 to -1226 bp region control the expression of the *atExt1*::*GUS* fusion gene in response to bacterial infection as well as wounding.

Analysis of the *atExt1* promoter sequence in combination with an analysis of promoter truncations linked to *GUS* has therefore identified a large number of putative *cis*-acting elements based on their similarity to known transcription factor binding sites. Although the position of many of these potential motifs matches up to their proposed function in the gene promoter, further research is required to confirm the activity and role of each of these elements.

References

Boyle, B., Brisson, N.: Repression of the defense gene *PR-10a* by the single-stranded DNA binding protein SEBF. - Plant Cell **13**: 2525-2537, 2001.

Brisson, L.F., Tenhaken, R., Lamb, C.: Function of oxidative cross-linking of cell wall structural proteins in plant disease resistance. - Plant Cell **6**: 1703-1712, 1994.

Cannon, M.C., Terneus, K., Hall, Q., Tan, L., Wang, Y., Wegenhart, B.L., Chen, L., Lamport D.T.A., Chen, Y., Kieliszewski M.J.: Self-assembly of the plant cell wall requires an extensin scaffold. - Proc. nat. Acad. Sci. USA **105**: 2226-2231, 2008.

Cassab, G.I.: Plant cell wall proteins. - Annu. Rev. Plant Physiol. Plant mol. Biol. **49**: 281-309, 1998.

Cheong, Y.H., Chang, H.-S., Gupta, R., Wang, X., Zhu, T., Luan, S.: Transcriptional profiling reveals novel interactions between wounding, pathogen, abiotic stress, and hormonal responses in *Arabidopsis*. - Plant Physiol. **129**: 661-677, 2002.

Clough, S.J., Bent, A.F.: Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. - Plant J. **16**: 735-743, 1998.

Corbin, D.R., Sauer, N., Lamb, C.J.: Differential regulation of a hydroxyproline-rich glycoprotein gene family in wounded and infected plants. - Mol. cell. Biol. **7**: 4337-4344, 1987.

Dröge-Laser, W., Kaiser, A., Lindsay, W., Halkier, B.A., Loake, G.J., Doerner, P., Dixon, R.A., Lamb, C.: Rapid stimulation of a soybean protein-serine kinase that phosphorylates a novel bZIP DNA-binding protein, G/HBF-1, during the induction of early transcription-dependent defenses. - EMBO J. **16**: 726-738, 1997.

Elliott, K.A.: The regulation of *extA*: an extensin gene from *Brassica napus*. - PhD Thesis. University of Wales, Bangor 1998

Elliott, K.A., Shirsat, A.H.: Promoter regions of the *extA* extensin gene from *Brassica napus* control activation in response to wounding and tensile stress. - Plant mol. Biol. **37**: 675-687, 1998.

Eulgem, T., Rushton, P.J., Robatzek, S., Somssich, I.E.: The WRKY superfamily of plant transcription factors. - Trends Plant Sci. **5**: 199-206, 2000.

Eulgem, T., Rushton, P.J., Schmelzer, E., Hahlbrock, K., Somssich, I.E.: Early nuclear events in plant defence signalling: rapid gene activation by WRKY transcription factors. - EMBO J. **18**: 4689-4699, 1999.

Gallagher, S.R.: GUS Protocols: Using *GUS* Gene as a Reporter of Gene Expression. - Academy Press, San Diego 1992.

Goldsbrough, A.P., Albrecht, H., Stratford, R.: Salicylic acid-inducible binding of a tobacco nuclear protein to 10 bp sequence which is highly conserved among stress inducible genes. - Plant J. **3**: 563-571, 1993.

Goodwin, S.B., Sutter, T.R.: Microarray analysis of *Arabidopsis* genome response to aluminum stress. - Biol. Plant. **53**: 85-99, 2009.

Guo, Y., Delseny, M., Puigdomenech, P.: mRNA accumulation and promoter activity of the gene coding for a hydroxyproline-rich glycoprotein in *Oryza sativa*. - Plant mol. Biol. **25**: 159-165, 1994.

Hamilton, D.A., Schwarz, Y.H., Mascarenhas, J.P.: A monocot pollen-specific promoter contains separable pollen-specific and quantitative elements. - Plant mol. Biol. **38**: 663-669, 1998.

Hauffe, K.D., Lee, S.P., Subramaniam, R., Douglas, C.J.: Combinatorial interactions between positive and negative *cis*-acting elements control spatial patterns of 4cl-1 expression in transgenic tobacco. - Plant J. **4**: 235-253, 1993.

Hirsinger, C., Salva, I., Marbach, J., Durr, A., Fleck, J., Jamet, E.: The tobacco extensin gene *Ext 1.4* is expressed in cells submitted to mechanical constraints and in cells proliferating under hormone control. - J. exp. Bot. **50**: 343-355, 1999.

Humphrey, T.V., Bonetta, D.T., Goring, D.R.: Sentinels at the wall: cell wall receptors and sensors. - New Phytol. **176**: 7-21, 2007.

Izawa, T., Foster, R., Chua, N.H.: Plant bZIP protein DNA binding specificity. - J. mol. Biol. **230**: 1131-1144, 1993.

Jefferson, R.A., Kavanagh, T.A., Bevan, M.W.: *GUS* fusions: *β-glucuronidase* as a sensitive and versatile gene fusion marker in higher plants. - EMBO J. **6**: 3901-3907, 1987.

Kawaoka, A., Sato, S., Nakahara, K., Matsushima, N., Okada, N., Sekine, M., Shinmyo, A., Takano, M.: Expression and promoter activity of genes for isozymes of horseradish-peroxidase. - Plant Cell Physiol. **33**: 1143-1150, 1992.

Kay, R., Chan, A., Daly, M., McPherson, J.: Duplication of CaMV-35S promoter sequences creates a strong enhancer for plant genes. - Science **236**: 1299-1302, 1987.

Klotz, K.L., Lagrimini, L.M.: Phytohormone control of the tobacco anionic peroxidase promoter. - Plant mol. Biol. **31**:

565-573, 1996.

Lamport, D.T.A.: Life behind cell walls: paradigm lost, paradigm regained. - Cell. mol. Life Sci. **58**: 1363-1385, 2001.

Lebel, E., Heifetz, P., Thorne, L., Uknes, S., Ryals, J., Ward, E.: Functional analysis of regulatory sequences controlling *PR-1* gene expression in *Arabidopsis*. - Plant J. **16**: 223-233, 1998.

Mason, H.S., DeWald, D.B., and Mullet, J.E.: Identification of a methyl jasmonate-responsive domain in the soybean vsp8 promoter. - Plant Cell **5**: 241-251, 1993.

Memelink, J., Swords, K.M.M., Dekam, R.J., Schilperoort, R.A., Hoge, J.H.C., Staehelin, L.A.: Structure and regulation of tobacco extensin. - Plant J. **4**: 1011-1022, 1993.

Menossi, M., Rabaneda, F., Puigdomenech, P., Martinez-Izquierdo, J.A.: Analysis of regulatory elements of the promoter and the 3' untranslated region of the maize *Hrgp* gene coding for a cell wall protein. - Plant Cell Rep. **21**: 916-923, 2003.

Merkouropoulos ,G.: Regulation and analysis of *atExt1*, a novel extensin gene from *Arabidopsis thaliana*. - PhD Thesis. School of Biological Sciences, University of Wales, Bangor 2000.

Merkouropoulos, G., Barnett, D.C., Shirsat, A.H.: The *Arabidopsis* extensin gene is developmentally regulated, is induced by wounding, methyl jasmonate, abscisic and salicylic acid, and codes for a protein with unusual motifs. - Planta **208**: 212-219, 1999.

Merkouropoulos, G., Shirsat, A.H.: The unusual *Arabidopsis* extensin gene *atExt1* is expressed throughout plant development and is induced by a variety of biotic and abiotic stresses. - Planta **217**: 356-366, 2003.

Meshi, T., Iwabuchi, M.: Plant transcription factors. - Plant Cell Physiol. **36**: 1405-1420, 1995.

Niebel, A., Engler, J.D., Tire, C., Engler, G., Van Montagu, M., Gheysen, G.: Induction patterns of an extensin gene in tobacco upon nematode infection. - Plant Cell **5**: 1697-1710, 1993.

Nishiuchi, T., Shinshi, H., Suzuki, K.: Rapid and transient activation of transcription of the ERF3 gene by wounding in tobacco leaves – possible involvement of NtWRKYs and autorepression. - J. biol. Chem. **279**: 55355-55361, 2004.

Ohme-Takagi, M., Shinshi, H.: Structure and expression of a tobacco *beta-1,3-glucanase* gene. - Plant mol. Biol. **15**: 941-946, 1990.

Pastuglia, M., Roby, D., Dumas, C., Cock, J.M.: Rapid induction by wounding and bacterial infection of an *S* gene family receptor-like kinase gene in *Brassica oleracea*. - Plant Cell **9**: 49-60, 1997.

Petersen, M., Brodersen, P., Naested, H., Andreasson, E., Lindhart, U., Johansen, B., Nielsen, H.B., Lacy, M., Austin, M.J., Parker, J.E., Sharma, S.B., Klessig, D.F., Martienssen, R., Mattsson, O., Jensen, A.B., Mundy, J.: *Arabidopsis* MAP kinase 4 negatively regulates systemic acquired resistance. - Cell **103**: 1111-1120, 2000.

Rouster, J., Leah, R., Mundyt, J., Cameron-Mills, V.: Identification of a methyl jasmonate-responsive region in the promoter of a lipoxygenase 1 gene expressed in barley grain. - Plant J. **11**: 513–523, 1997.

Roberts, K., Shirsat, A.H.: Increased extensin levels in *Arabidopsis* affect inflorescence stem thickening and height. - J. exp. Bot. **57**: 537-545, 2006.

Rushton, P.J., Reinstadler, A., Lipka, V., Lippok, B., Somssich, I.E.: Synthetic plant promoters containing defined regulatory elements provide novel insights into pathogen- and wound-induced signaling. - Plant Cell **14**: 749-762, 2002.

Rushton, P.J., Somssich, I.E.: Transcriptional control of plant genes responsive to pathogens. - Curr. Opin. Plant Biol. **1**: 311-315, 1998.

Sambrook, J., Fritsch, E.F., Maniatis, T.: Molecular Cloning: a Laboratory Manual (2nd Ed.). - Cold Spring Harbor Laboratory Press, New York 1989.

Sasaki, K., Ito, H., Mitsuhara, I., Hiraga, S., Seo, S., Matsui, H., Ohashi, Y.: A novel wound-responsive *cis*-element, VWRE, of the vascular system-specific expression of a tobacco peroxidase gene, *tpoxN1*. - Plant mol. Biol. **62**: 753-768, 2006.

Savenstrand, H., Brosche, M., Strid, A.: Regulation of gene expression by low levels of ultraviolet-B radiation in *Pisum sativum*: isolation of novel genes by suppression subtractive hybridisation. - Plant Cell Physiol. **43**: 402-410, 2002.

Schenk, P.M., Kazan, K., Wilson, I., Anderson, J.P., Richmond, T., Somerville, S.C., Manners, J.M.: Coordinated plant defense responses in *Arabidopsis* revealed by microarray analysis. - Proc. nat. Acad. Sci. USA **97**: 11655-11660, 2000.

Sessa, G., Morelli, G., Ruberti, I.: The ATHB-1 and -2 HD-ZIP domains homodimerise forming complexes of different DNA binding specificities. - EMBO J. **12**: 3507-3517, 1993.

Shirsat, A.H., Wieczorek, D., Kozbial, P.: A gene for *Brassica napus* extensin is differentially expressed on wounding. - Plant mol. Biol. **30**: 1291-1300, 1996.

Showalter, A.M., Bell, J.N., Cramer, C.L., Bailey, J.A., Varner, J.E,, Lamb, C.J.: Accumulation of hydroxyproline-rich glycoprotein mRNAs in response to funga1 elicitor and infection. - Proc. nat. Acad. Sci. USA **82**: 6551-6555, 1985.

Solano, R., Nieto, C., Avila, J., Canas, L., Diaz, I., Pazares, J.: Dual DNA binding specificity of a petal epidermis-specific MYB transcription factor (*MYB.Ph3*) from *Petunia hybrida*. - EMBO J. **14**: 1773-1784, 1995.

Tire, C., Derycke, R., Deloose, M., Inze, D., Van Montagu M., Engler, G.: Extensin gene expression is induced by mechanical stimuli leading to local cell wall strengthening in *Nicotiana plumbaginifolia*. - Planta **195**: 175-181, 1994.

Wei, G., Shirsat, A.H.: Extensin over-expression in *Arabidopsis* limits pathogen invasiveness. - Mol. Plant Pathol. **7**: 579-592, 2006.

Wingate, V.P.M., Ryan, C.A.: Uniquely regulated proteinase inhibitor I gene in a wild tomato species: inhibitor I family gene is wound-inducible in leaves and developmentally regulated in fruit. - Plant Physiol. **97**: 496-501, 1991.

Wycoff, K.L., Powell, P.A., Gonzales, R.A., Corbin., D.R., Lamb, C., Dixon, R.A.: Stress activation of a bean hydroxyproline-rich glycoprotein promoter is superimposed on a pattern of tissue-specific developmental expression. - Plant Physiol. **109**: 41-52, 1995.

Yamada, T., Sriprasertsak, P., Kato, H., Hashimoto, T., Shimizu, H., Shiraishi, T.: Functional-analysis of the promoters of phenylalanine ammonia-lyase genes in pea. - Plant Cell Physiol. **35**: 917-926, 1994.

Yamada, T., Tanaka, Y., Sriprasertsak, P., Kato, H., Hashimoto, T., Kawamata, S., Ichinose, Y., Shiraishi, T., Oku, H.: Phenylalanine ammonia-lyase genes from *Pisum sativum*: structure, organ-specific expression and regulation by fungal elicitor and suppressor. - Plant Cell Physiol. **33**: 715-725, 1992.

Yu, D., Chen, C., Chen, Z.: Evidence for an important role of WRKY DNA binding proteins in the regulation of NPR1 gene expression. - Plant Cell **13**: 1527–1539, 2001.