# *Arabidopsis thaliana* class IV chitinase is early induced during the interaction with *Xanthomonas campestris*

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**Abstract Endochitinases are widely distributed among higher plants, including a number of important crop species. They are generally considered to be involved in plant defence against potential pathogens. We have cloned a class IV chitinase gene (*AtchitIV*) from *Arabidopsis thaliana*. Southern blot analysis allowed the detection of two cross-hybridising genes in the *A. thaliana* genome. AtchitIV transcripts are detected in seedpods, but not in roots, inflorescence stems, leaves and flowers of healthy plants. The transcripts accumulated very rapidly in leaves after inoculation with *Xanthomonas campestris*. Maximum mRNA accumulation was reached one hour after infection and decreased to very low levels 72 hours after induction. This result suggests an involvement of AtchitIV in the initial events of the hypersensitive reaction. Nevertheless, *A. thaliana* plants transformed with the *gus* gene under the control of a class IV chitinase bean promoter, showed GUS activity in seed embryos. These data, together with the constitutive expression of the endogenous gene in the seedpods, points to additional physiological roles for this protein.**

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*Key words:* Class IV chitinase; Stress-induced protein; Gene expression; *Arabidopsis thaliana*

## 1. Introduction

The mechanism of plant protection against pathogen attack involves, among other metabolic modifications, the synthesis of hydrolases such as chitinases and glucanases [1]. Endochitinases (EC 3.2.1.14) catalyse the hydrolysis of chitin, a homopolymer of β-1,4-*N*-acetyl-D-glucosamine, which represents the major component of fungal cell walls and arthropod exoskeleton [2,3].

Collinge et al. [4] have proposed the categorisation of chitinases into four classes, based on the analysis of their sequences and domains. Class I, II and IV chitinases have a homologous catalytic domain. Enzymes from class I and IV have a cysteine-rich domain in their N-terminal region that is absent in class II chitinases. This cysteine-rich domain has been shown to be essential for substrate binding, but not for catalytic or antifungal activity [5]. In contrast to secreted chitinases (corresponding to all class II and IV proteins, and even some class I members), class I enzymes have a C-terminal peptide of about six amino acids targeting the protein for vacuolar localisation [6]. The class III chitinases are bifunctional lysozyme/chitinase enzymes and do not share any sequence similarity with plant chitinases from other classes. The class IV corresponds to a group of extracellular chitinases with two characteristic deletions in the main catalytic domain [4]. Other proteins with endochitinase activity were isolated from tobacco [7]. Because they share some similarities with bacterial exochitinases, but are unrelated to the plant chitinases previously described, they fall in a new group, the class V chitinase.

Due to their potential antimicrobial activity, chitinases have received much attention. These enzymes are present in all plants analysed to date and it has been demonstrated that these proteins, alone or acting synergistically with glucanases, can inhibit fungal growth either in vitro or in vivo when overexpressed in transgenic plants [3,8–11]. Chitinases are found in low amounts in healthy plants, but increased levels of gene expression have been observed in leaves after inoculation with fungi [12], bacteria [13] or viruses [14]. The chitinase involvement in the nodulation process has also been reported [15–17]. Additionally, these enzymes can be induced by wounding [14], heat shock [18], ethylene [19], fungal elicitors [20] or chemicals such as salicylic acid, mercuric chloride [21] or lead nitrate [22]. Apart from the response to environmental factors, some plant chitinases are produced during certain stages of the plant development and therefore their role in the normal cellular metabolism must be considered. In common bean (*Phaseolus vulgaris*) plants, the P4-ch, a class IV chitinase, is induced during germination and is expressed in roots of mature plants [18]. Furthermore, high expression of chitinase and glucanase in differentiating healthy tobacco flowers has been reported [23,24]. A 32 kDa class IV chitinase, secreted by wild-type carrot cells, can rescue a carrot mutant in somatic embryo formation [25,26]. These data suggest that some chitinases may participate in the early stages of embryo development.

The system *Arabidopsis thaliana* infected by *Xanthomonas campestris* was used here as a model to obtain a better insight into the early events of plant responses against pathogen attacks. The previous work of Lummerzheim et al. [13] identified both compatible and incompatible interactions between *A. thaliana* and *X. campestris*. The authors showed that transcripts hybridising with a bean basic class I chitinase cDNA accumulated in *A. thaliana* during compatible and incompatible interactions. Up to date, two classes of chitinases have been identified in *A. thaliana*. A basic class I chitinase local-

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ised in vacuoles, and an extracellular acidic class III enzyme [27]. The basic chitinase displays age-dependent and tissue-specific expression and is induced by ethylene treatment. In this paper, we report the molecular cloning and characterisation of an *A. thaliana* class IV chitinase gene. Chitinase transcript accumulation in the different tissues of healthy plants, as well as the temporal expression pattern in leaves during an incompatible interaction with *X. campestris* are also analysed.

## 2. Materials and methods

### 2.1. Plant material and bacteria inoculation procedure

*A. thaliana* plants ecotype Columbia-O were grown from seeds under controlled conditions in a growth chamber at 22°C with a relative humidity of 75% and a daily period of 12 h. Roots, inflorescence stems, leaves, flowers and seedpods were collected from 5 to 6 week-old plants. Plants at the same age were inoculated with the bacteria *X. campestris* pv. *campestris* (strain 147) [13]. The bacteria were routinely grown on Kado medium [28] at 28°C. The inoculation of the bacterial suspension in sterile water ($OD_{600nm} = 0.3$) was made by infiltration beneath the leaf, using a disposable 1 ml syringe as previously described [13]. As control, the leaves were inoculated with sterile water. The leaves were harvested at various times after inoculation.

### 2.2. Screening of an A. thaliana genomic library, cloning and analysis of the chitinase genes

The *A. thaliana* (ecotype Columbia) genomic library constructed in the cosmid vector pC22 [29] was screened by hybridisation of about $1 \times 10^5$ colonies with [$^{32}$P] labelled bean P4 chitinase cDNA [30] and, in a second screening, with the *Eco*RI/*Bam*HI fragment of the 8.2.1 *A. thaliana* genomic clone, isolated in the first screening. The hybridisation was performed according to standard procedures [31]. Positive clones were subjected to additional rounds of screening at low colony density. The restriction mapping of the positive clones was conducted by Southern hybridisation of restriction DNA fragments, using as probes, initially the bean P4 chitinase cDNA and after that, the *Eco*RI/*Bam*HI fragment of 8.2.1 *A. thaliana* genomic clone we have isolated. The DNA fragments hybridising to the probes were subcloned either into pBluescript II (Stratagene) or pGEM-2 (PROMEGA) and sequenced. The sequence analysis was performed using PC/gene (Intelligenetics), Transcription Element Search Software (TESS) on the WWW [32] and BCM Search Launcher (http://gc.bcm.tmc.edu:8088/search-launcher/launcher.html). Similarity dendrogram tree construction was conducted using the MEGA analysis platform [33]. The genomic sequence of *AtchitIV* appears in the EMBL data bank under accession number Y14590.

### 2.3. Southern and Northern blot analysis

Genomic DNA was isolated from leaves of *A. thaliana* according to Dellaporta et al. [34]. About 20 μg of DNA was digested with appropriate restriction enzymes and separated on a 1% agarose gel. At specific intervals of time after *X. campestris* inoculation, total RNA was isolated from leaves of *A. thaliana* according to Ragueh et al. [35]. For analysis of tissue-specific expression, total RNA was isolated from different parts of the plant using the same method. RNA samples (20 μg) were fractionated by electrophoresis on formaldehyde-containing agarose gels. Southern and Northern blots were performed according to Sambrook et al. [31], using an *Eco*RI/*Bam*HI fragment of the clone 8.2.1 as probe.

### 2.4. Construction of P4-ch promoter-gus fusion, plant transformation and histochemical GUS assay

The fusion of the bean P4 chitinase promoter region to the *gus* gene was obtained by introduction, by PCR mutagenesis, of an *Nco*I site in the chitinase ATG region of the clone pGPR45 [36,37]. The primers 5′ AATTTGTTTCCCATGGTGAAGG 3′ and 5′ GATATCGTCGACCACAAATATGTTTCAGGTTA 3′ were employed in this experiment. The 600 bp PCR product was digested with *Sal*I and *Nco*I and subcloned into pGUSI vector (Plant Genetic System N.V., Belgium). This chimeric gene, with 600 bp 5′ upstream region of the bean P4 chitinase controlling *gus* expression, was cloned into the *Eco*RI/*Hin*dIII sites of the plant transformation vector pDE1001 (Plant Genetic Systems) to give pΔ600GP4. *A. thaliana* ecotype C-24 and *Nicotiana tabacum* cv. SR1 plants were transformed with pΔ600GP4 via *Agrobacterium tumefaciens* as described by Valvekens et al. [38] and Horsch et al. [39], respectively.

Histochemical GUS assay was done according to Jefferson [40] with minor modifications described in Sachetto-Martins et al. [41].

## 3. Results and discussion

### 3.1. Isolation and nucleotide sequence analysis of AtchitIV gene

The *A. thaliana* genomic library was screened with the bean class IV chitinase cDNA (*P4-ch*) [30], and one positive clone was obtained (8.2.1). The analysis of the 1.16 kb *Eco*RI/*Bam*HI fragment of this clone revealed a sequence of 195 bp with 78.46% of identity to the initial 200 bp of the second exon of bean *P4-ch* gene [18]. The remaining 965 bp has no homology with any chitinase. Further restriction analysis revealed that the genomic clone terminates at the *Bam*HI site in the second exon, therefore it was incomplete at the 3′ end.

A second screening of the *A. thaliana* genomic library was then carried out using the *Eco*RI/*Bam*HI fragment of the clone 8.2.1 as probe. The use of this homologous probe allowed the identification of nine candidates. The Southern blot analysis of these clones digested with different endonucleases (*Eco*RI, *Bam*HI, *Bgl*II, *Hin*dIII, *Pst*I), showed the same restriction pattern, indicating that they may correspond to the same gene (data not shown). One clone (16.1) was chosen for further characterisation. The restriction fragments *Eco*RI/*Bam*HI, *Eco*RI/*Pst*I, *Hin*dIII/*Bam*HI and *Pst*I/*Bam*HI, with the estimated sizes of 0.6, 2.1, 0.8, and 1.9 kb respectively, were subcloned into pGEM-2 vector and their sequence determined.

Fig. 1 shows the complete nucleotide sequence of the genomic clone 16.1, deduced from the sequencing of the subclones as showed in Fig. 2. The analysis of the 5′ flanking region of *AtchitIV* revealed putative TATA- and CAAT-boxes at positions 1009–1015 and 940–943, respectively (Fig. 1). No consensus polyadenylation signal sequence AATAAA was found within the sequenced 3′ region. A search for regulatory elements within the promoter region of the *AtchitIV* revealed the presence of sequences homologous to putative *cis*-acting elements (Table 1). At least two of them are binding sites for

Table 1
Putative *cis*-acting elements identified in the AtchitIV promoter region and its proposed function

| *cis*-element | Position | Proposed *trans*-acting factor | Reference |
|---|---|---|---|
| ACGTCA (reverse-complementar) | 393–398 | HBP(s) are involved in cell cycle-dependent transcription of the *H3* gene (*Triticum aestivum*) | Mikami et al. [42] Mikami et al. [43] Tabata et al. [44] |
| YAAAAAY (reverse-complementar) | 641–647 | SEF-4 is involved in transcriptional regulation of β-*conglycinin* gene regulated during embryogenesis (*Glycine max*) | Lessard et al. [45] |
| YCAYRTCAtC (reverse-complementar) | 391–400 | Opaque-2 is the factor responsible for the transcriptional activation of *zeins* genes (*Zea mays*) | Schmidt et al. [46] |

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   1 ctgcagatcttcctggtgactatatatgtcttcaaggagactttcaagtttcatttcttt
  61 acaagaatgttactctctttacctcacctccacaaaagtaagctgtttactattaacaac
 121 ctccgataccgctgttctcagcttcttaaagtttctgttgttgtctccgacgaaaacatt
 181 agttcgattggaacatacattatggaagatgtcaaagagaagaagcaagtaacttttgga
 241 ttgcagttttctcttaccgattgcagaaaaaagacgttaggagttatgagttatgcatgc
 301 tgtggagtcaccttgaggttcgaacccggatctgagatgaaggcgtctgtgtttggaaag
 361 catccaaggtacacaaatttctgaccaagtccacgtcaacaccagtataatgttagagtt
 421 tgcaaagatttgacattacttaagggctttattgaattagagatttagtttatttgtaaa
 481 tgactttagatttggttcgactttatttagggtctaaagtttattgtataactgtttaat
 541 atttgtcaggacttaatgaagatgtaatgtttagactttggtgtattttgtacgacatgc
 601 atgatataataaaggaattatcataattaaattgaacttataaaaatatgacttgcctta
 661 cttctcttttgaaatagaaattctaatgtaggtaaaatgtttgtgtttacataattacat
 721 tacatcaacaaacgtaaaaaaatgtttgtgtcattaaatgtttatccacgtactcttcac
 781 aacgatcataagtcataattcagaaaattcctttaataaatgtgaactgaaaaagctttt
 841 gtatagtcatcaaataaaaagtcaactgtacaattaaaagtccgcaaacatctcgatcgt
 901 ctaagagacgacttttctcgtaactttatgaagaatatacaatagtagtggcttgatcgc
 961 gtccacacatttacattgcgacttttcttgcatgccgtctcacgcactatatttaggcaa
1021 aacacaattgtcttcatttcaagactagttcttgttttctccatattcctcaacaacatc
1081 aaaatgttgactcccaccatttctaaatccatctctttagtaaccattctattagttcta
        M  L  T  P  T  I  S  K  S  I  S  L  V  T  I  L  L  V  L
1141 caagctttctctaacacaacaaaggctcaaaattgcggttgttcgtcagagctatgttgt
     Q  A  F  S  N  T  T  K  A  Q  N  C  G  C  S  S  E  L  C  C
1201 agtcagtttggcttttgcggtaacacttcagactattgtggtgtaggttgccaacaagga
     S  Q  F  G  F  C  G  N  T  S  D  Y  C  G  V  G  C  Q  Q  G
1261 ccttgttttgctcctccccctgcaaatggtgtctctgtggctgagattgtaacgcaagaa
     P  C  F  A  P  P  P  A  N  G  V  S  V  A  E  I  V  T  Q  E
1321 ttcttcaatggaatcatcagtcaagccgcgtctagttgcgccggcaatagattttacagt
     F  F  N  G  I  I  S  Q  A  A  S  S  C  A  G  N  R  F  Y  S
1381 cggggagcttttcttgaggccttagactcatattctcgtttcggtagagttggatcgacc
     R  G  A  F  L  E  A  L  D  S  Y  S  R  F  G  R  V  G  S  T
1441 gacgactctaggcgtgagattgcagcgttctttgctcatgtcacacatgaaacaggacgt
     D  D  S  R  R  E  I  A  A  F  F  A  H  V  T  H  E  T  G  H
1501 aagtatatatagtaacatgtttcactttactcttttctttatgatgcatacatgcatgtt
1561 ctctattaaataccaaacaaaaacaaaatatgaaaacgttagaacttgtttttgttttct
1621 aaaaatcattagactaaataacatgttttactagattagtaatatcttttcatcaaacgc
1681 atatagcataatcacttttcaaaatcatagaaacttgactagaaatactttgaatatgtt
1741 gcagcattcgatctaataatttcgttttttgttttagatttctgctacatagaagagata
                                                  F  C  Y  I  E  E  I
1801 gacggagcctcaaaggattactgcgacgagaatgcaacacaatatccatgcaatcctaac
     D  G  A  S  K  D  Y  C  D  E  N  A  T  Q  Y  P  C  N  P  N
1861 aaaggctactacggccgcggaccgatccaactctcttggaatttcaactacgggccagcc
     K  G  Y  Y  G  R  G  P  I  Q  L  S  W  N  F  N  Y  G  P  A
1921 gggacagcaattggtttcgacggcctgaatgcaccggaaacagtagccacggatccagtc
     G  T  A  I  G  F  D  G  L  N  A  P  E  T  V  A  T  D  P  V
1981 atatccttcaaaaccgccttgtggtactggaccaatagggttcagcctgttatctctcaa
     I  S  F  K  T  A  L  W  Y  W  T  N  R  V  Q  P  V  I  S  Q
2041 ggttttggtgcaacaatccgtgccattaacggtgctttggagtgtgacggggccaacaca
     G  F  G  A  T  I  R  A  I  N  G  A  L  E  C  D  G  A  N  T
2101 gccaccgttcaagctagagttcgttactacacggattattgtcgtcaacttggcgttgat
     A  T  V  Q  A  R  V  R  Y  Y  T  D  Y  C  R  Q  L  G  V  D
2161 cctggaaacaacctcacttgctaacaaaaacatctatttctctgtatacatatataaaag
     P  G  N  N  L  T  C  *
2221 taaaaatagacaagctttaagaatggaatggcccattaatgttttgtcccttaatgtcga
2281 atatatatgaaatccacttagttaaaaaaagacaaaaactttgagatgaaaacagagaaa
2341 ctttatacacagaatcctatgaagtagtttccatcattttt
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Fig. 1. Nucleotide sequence of the *AtchitIV* gene. Putative TATA- and CAAT-boxes are underlined. The intron region is in italic and the stop codon is indicated by an asterisk. The deduced amino acid sequence is in upper-case letter.

transcriptional factors (SEF-4 and Opaque-2) involved in the embryogenic process. The significance and functionality of these putative *cis*-elements needs further studies.

This gene is organised in two exons of 415 bp (exon 1) and 404 bp (exon 2) separated by an intronic sequence of 279 bp. The intron is AT rich (74.9%) and contains a consensus splice junction (5′ GT at position 1499 and 3′ AG at position 1777 relative to the beginning of the clone) [47,48]. Furthermore, like in mammals, this intron has, preceding the 3′ splice site, an extended pyrimidine tract that is often absent in plant genes. The analysis of this sequence also revealed another consensus 3′ splice junction 38–34 bp upstream the first 3′ splice site. This alternative splice contains a conserved G at −4 and a conserved C at −3 in the intron/exon 2 boundary [49]. Considering the 3′ AG at position 1777 as the functional splicing site, the *A. thaliana* chitinase gene contains a single intron at the same position of the bean P4 chitinase. It has a complete open reading frame of 819 bp and encodes a

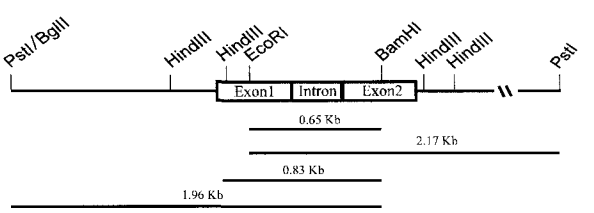


Fig. 2. Schematic representation of the organisation and restriction mapping of the genomic clone *AtchitIV* and the fragments *Eco*RI/*Bam*HI, *Eco*RI/*Pst*I, *Hin*dIII/*Bam*HI and *Pst*I/*Bam*HI used for subcloning and sequencing.

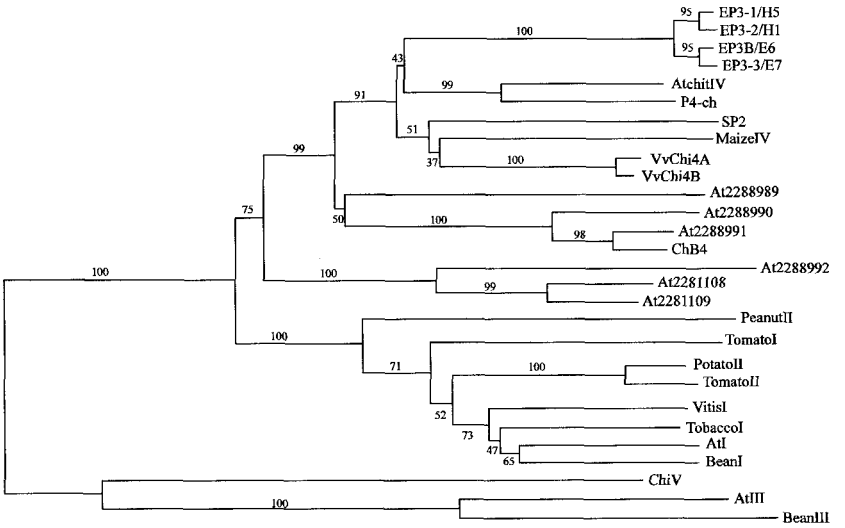


Fig. 3. Similarity dendrogram among AtchitIV and 27 chitinases related sequences. Bean P4-ch (X57187), *Brassica napus* ChB4 (X61488), carrot EP3 1/H5 (U52845), EP3 2/H1 (U52846), EP3 3B/E6 (U52848) and EP3 3/E7 (U52847), grape VvCh4A (U97521) and VvCh4B (U97522), maize maizeA (M84164), sugar beet SP2 (L25826), *A. thaliana* chromosome II: At2281108, At2281109, At2288989, At2288990, At2288991 and At2288992 (AC002333; AC002335), PeanutII (X82329), TomatoII (U30465), PotatoII (X67693), TomatoI (Z15139), VitisI (Z54234), TobaccoI (X64519), BeanI (M13968), *A. thaliana* class I (AtI) (M38240) and class III (AtIII) (M34107), BeanIII [56] and ChiV (X77111). The figures between brackets correspond to GenBank accession no.

polypeptide of 273 amino acid residues. Deletions in the catalytic domain, characteristic to all class IV chitinases, are also present in the protein encoded by this genomic clone, indicating that it belongs to chitinases of the class IV. However, if the alternative splicing is real, the primary transcript processing could result in a protein without the first deletion in the catalytic domain. The hypothesis of an alternative splicing will be further investigated.

The deduced protein has an estimated isoelectric point of 4.8 and a calculated molecular mass of 30 kDa. The N-terminal region is constituted of hydrophobic amino acids and shares similarity to known signal peptide sequences. However the carboxy-terminal extension for targeting to plant vacuole [6] is not present, suggesting an extracellular location.

Studies of primary structure comparison and homologies show a high degree of identity between the *A. thaliana* chitinase and class IV chitinases previously described in other plant species (cowpea 71.1% [50], common bean 70.9% [51], grape 68.6% [52], *Brassica napus* 59.7% [53], carrot 59.0% [26], sugar beet 56.4% [54] and maize 56.0% [55]). These data support the hypothesis that the genomic clone 16.1 corresponds to an *A. thaliana* class IV chitinase (*AtchitIV*) gene. Several new chitinase isolog genes have been recently identified in *A. thaliana* through systematic sequencing of the chromosome II (GenBank accession nos. AC002333 and AC002335). All of them display deletions in the catalytic domain characteristic of class IV chitinases. The putative translated sequence of the genes 2288991 and 2288990 are 59.47% and 56.23% identical to the AtchitIV, respectively, while 2288989, 2281108, 2281109 and 2288992 display lower identity: 48.35%, 45.42%, 43.59% and 32.23%, respectively. In spite of this low degree of similarity, the dendrogram analysis shows that they belong to class IV chitinase cluster (Fig. 3). Although derived from the same species, AtchitIV is closer related to bean chitinase IV and carrot chitinases active in somatic embryos than to *A. thaliana* chromosome II chitinases.

The cloning of a class IV chitinase gene in *A. thaliana* is in agreement with the recent propositions of Hamel et al. [57] who have remarked that in some plant species such as *Beta vulgaris*, *P. vulgaris* and *Zea mays*, the presence of class IV chitinases fulfils the need for extracellular enzymes. According to these authors, the class IV chitinases seem to have been replaced or complemented in various plants by other types of extracellular chitinases. As examples, in solanaceae *N. tabacum* and *Lycopersicon esculentum*, several class II but not class IV chitinases have been isolated. In contrast, in bean and *A. thaliana* only intravacuolar class I and extracellular class III and IV but not class II chitinases have been identified [14,27].

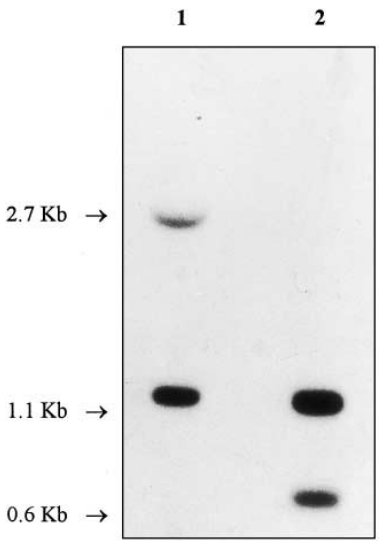


Fig. 4. Southern blot of *Arabidopsis thaliana* genomic DNA probed with the *Eco*RI/*Bam*HI fragment of the clone 8.2.1. Total DNA was digested with *Eco*RI (1) and *Eco*RI/*Bam*HI (2) restriction enzymes, separated by electrophoresis in a 1.0% agarose gel, blotted to nylon membrane, and hybridised with the probe.

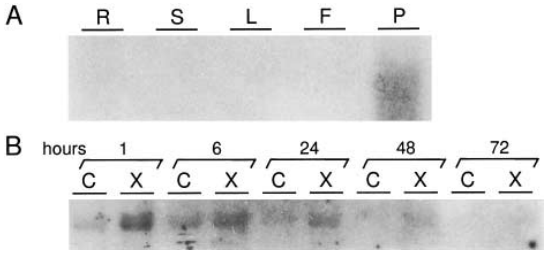


Fig. 5. Northern analysis. A: RNA was extracted from roots (R), inflorescence stems (S), leaves (L), flowers (F) and seedpods (P); B: RNA from leaves infiltrated with *Xanthomonas campestris* (X) at different hours after inoculation compared to water control (C) at the same time points. The filters were probed with the *Eco*RI/*Bam*HI fragment of the clone 8.2.1.

### 3.2. Genomic Southern hybridisation

In preliminary studies on cloning *AtchitIV*, the number of genes was determined by Southern blot. For this analysis 20 μg of *Arabidopsis* genomic DNA were digested with either *Eco*RI or *Eco*RI/*Bam*HI restriction endonucleases. The Southern blot hybridisation was performed with the labelled *Eco*RI/*Bam*HI fragment of the clone 8.2.1. The digestion with *Eco*RI/*Bam*HI (lane 2) revealed two hybridising bands of about 1.1 kb fragment and 0.6 kb (Fig. 4), indicating that there are at least two genes encoding AtchitIV. The 1.1 kb *Eco*RI/*Bam*HI fragment corresponds to the clone 8.2.1, isolated during the first screening, and the 0.6 kb *Eco*RI/*Bam*HI fragment corresponds to the clone 16.1, in agreement with our sequencing data. This probe did not cross-hybridise to any of the *A. thaliana* chitinase genes described elsewhere. The sequence comparison between the clones 16.1 and 8.2.1 revealed that both are identical at their coding sequence but have very different intron regions. In the clone 8.2.1, a segment of 965 bp 5′ upstream the exon 2 shows no similarity with intron or exon 1 sequences of the clone 16.1. Two hypothesis can be raised from this result: the clone 8.2.1 corresponds either to a pseudogene or to another functional chitinase gene with a longer and not related intronic region.

### 3.3. Chitinase expression analysis

Northern blot hybridisation was used to monitor the expression of the *AtchitIV* gene in *Arabidopsis* plants at different times (1, 6, 24, 48 and 72 h) after inoculation with the bacteria *X. campestris*. The Northern blot was performed with 20 μg of total RNA probed with the *Eco*RI/*Bam*HI fragment of the clone 8.2.1. As shown in Fig. 5B, a maximal induction of chitinase mRNA occurs already 1 h after the inoculation. The transcript levels gradually decrease in the following hours post-inoculation, and is not detectable after 72 h. The weak signal detected in the control plants is probably due to the wounding caused by the inoculation procedure, since no transcript was detected in uninfected leaves (Fig. 5A). The probe hybridised to transcript species of 1.1 kb. Similar results were obtained by probing with the *Eco*RI/*Bam*HI fragment of the clone 16.1 (data not shown). Because the probe derived from the clone 8.2.1 can hybridise with both genes, the origin of the transcripts detected in the Northern blot remains unclear. Also, both genes may respond similarly during the plant-pathogen interaction.

The accumulation of chitinase mRNAs indicates that these enzymes might be part of the defence mechanisms against pathogen attacks. The fact that the induction of transcripts occurs very early in the response may suggest that this chitinase could also perform signalling functions, releasing elicitors for the establishment of a hypersensitive reaction.

RNA was also isolated from different organs of a healthy *Arabidopsis* plant in order to analyse the *AtchitIV* chitinase expression pattern. The Northern analysis showed high levels of transcripts in the seedpods but no expression was detected in roots, inflorescence stems, leaves and flowers (Fig. 5A). This result may indicate that this class IV chitinase could be involved in either seed or embryo development. This data, and the very strong structural similarity between AtchitIV and bean P4 chitinase (P4-*ch*) [18], prompted us to investigate the expression of the P4-*ch* promoter in transgenic *A. thaliana* and tobacco plants. As shown in Fig. 6, 600 bp of P4-*ch* promoter region confers *gus* expression in the embryos of *A. thaliana* (Fig. 6A) and tobacco (Fig. 6B). In zygotic embryos of transgenic *Arabidopsis*, GUS activity was observed at the heart shaped and torpedo stage. No expression was observed in other seed tissues (Magioli et al., in preparation).

Class IV chitinase and Nod factors can rescue an embryogenesis-defective mutant (ts11) [25,26,58]. Nod factors are lipo-chitooligosaccharides that resemble the fungal chitin elicitors and can be further degraded by chitinases [59,60]. These results show that chitinase activity plays an important role during embryogenesis. de Jong et al. [58] suggested that cell wall modifications may be an essential step during the embryo development and that chitinases may be involved on this reorganisation. Since ts11 mutants are arrested at the globular stage, the class IV chitinase may act liberating or degrading signals involved in the transition from the globular to heart shape embryo.

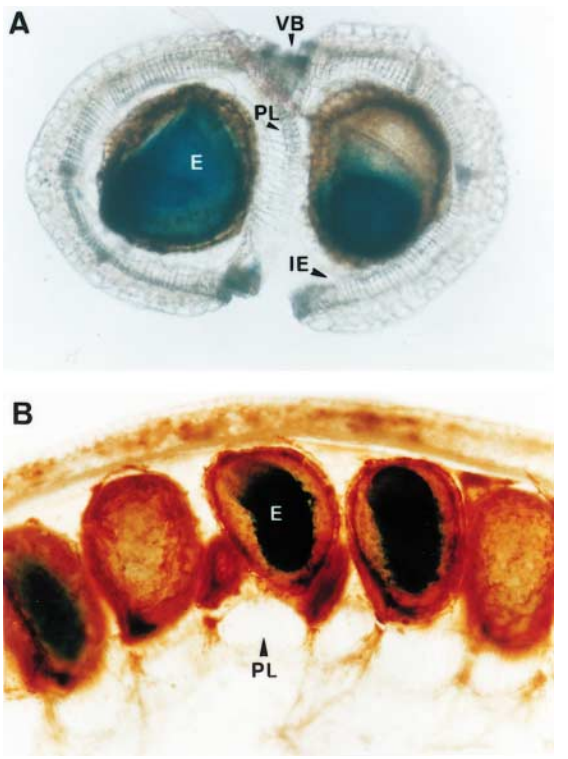


Fig. 6. Expression of the construction pΔ600GP4 in fruits of transgenic *A. thaliana* (A) and tobacco (B) plants using histochemical detection of GUS activity. VB, vascular bundle; PL, placenta; IE, inner epidermis; and E, embryo.

Chitinases are also expressed in different types of health tissues, such as roots [18] and mature flowers [23,24], and during other developmental processes such as germination [18], senescence [61], and nodulation [15–17]. It is possible that chitinases release cell wall factors that may be responsible for the induction of specific developmental changes, similarly as proposed for embryo development.

The specific physiological role of class IV chitinase in plants remains unclear. Our results indicate that class IV chitinase genes are involved in both plant-pathogen interactions and embryo development. The cloning and characterisation of the *A. thaliana* class IV chitinase gene reported here open the possibility to address important questions about the specific functions of these hydrolases in plants.

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