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# *Arabidopsis Chitinases*: a Genomic Survey

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**Abstract. Plant chitinases (EC 3.2.1.14) belong to relatively large gene families subdivided in classes that suggest class-specific functions. They are commonly induced upon the attack of pathogens and by various sources of stress, which led to associating them with plant defense in general. However, it is becoming apparent that most of them display several functions during the plant life cycle, including taking part in developmental processes such as pollination and embryo development. The number of chitinases combined with their multiple functions has been an obstacle to a better understanding of their role in plants. It is therefore important to identify and inventory all chitinase genes of a plant species to be able to dissect their function and understand the relations between the different classes. Complete sequencing of the Arabidopsis genome has made this task feasible and we present here a survey of all putative chitinase-encoding genes accompanied by a detailed analysis of their sequence. Based on their characteristics and on studies on other plant chitinases, we propose an overview of their possible functions as well as modified annotations for some of them.**

## 1. Introduction

Chitinases (EC 3.2.1.14) are classified as glycosyl hydrolases and catalyze the degradation of chitin, an insoluble linear b-1,4-linked polymer of N-acetyl-D-glucosamine (GlcNAc). Chitin is a major component of the exoskeleton of insects, of crustacean shells and of the cell wall of many fungi. According to the glycosyl hydrolase classification system that is based on amino acid sequence similarity of the catalytic domains, chitinases have been placed in families 18 and 19 (Henrissat, 1991). Family 18 chitinases are found in bacteria, fungi, yeast, viruses, plants and animals whereas family 19 members are almost exclusively present in plants. A single family 19 chitinase was identified in *Streptomyces griseus* (Ohno *et al*., 1996; Watanabe *et al*., 1999). Chitinases of both families do not share sequence similarity and have a different 3D-structure, suggesting that they have arisen from a different ancestor (Hamel *et al*., 1997). They also differ in several of their biochemical properties. For instance, family 18 chitinases use a retention mechanism, keeping the catalysis product in the same configuration as the substrate (i.e. b-anomeric form) whereas family 19 members use an inversion mechanism turning the product into the a-anomeric form (Brameld and Goddard, 1998; Iseli *et al*., 1996). In addition, family 18 members hydrolyze GlcNAc-GlcNAc or GlcNAc-GlcN linkages whereas family 19 chitinases do so with GlcNAc-GlcNAc or GlcN-GlcNAc linkages (Ohno *et al*., 1996). Finally, family 18 chitinases are likely to function according to a substrate-assisted catalysis model (Brameld *et al*., 1998), whereas family 19 chitinases probably use a general acid-and-base mechanism (Garcia-Casado *et al*., 1998; Hart *et al*., 1995).

In all plants analyzed to date, chitinases of both families are present (Graham and Sticklen, 1994). They are organized in five different classes numbered from I to V,

according to their sequences and structure (Neuhaus *et al*., 1996) and chitinases from classes I, II and IV belong to the family 19 whereas classes III and V chitinases are made of family 18 chitinases. Chitinases are often considered as pathogenesis-related (PR) proteins, since their activity can be induced by fungal, bacterial and viral infections, but also by more general sources of stress such as wounding, salicylic acid, ethylene, auxins and cytokinins, heavy metal salts or elicitors such as fungal and plant cell wall components (reviewed in Graham and Sticklen, 1994). Plants do not contain chitin in their cell walls, whereas major agricultural pests such as most fungi (i.e. Ascomycetes, Basidiomycetes and Deuteromycetes; Collinge *et al.*, 1993) and insects do, leading to the obvious and often quoted hypothesis that chitinases act as a defense mechanism against pathogens. Evidence has been reported that chitinases can indeed degrade fungal cell walls and inhibit fungal growth in vitro, especially in combination with b-1,3-glucanases (Arlorio *et al*., 1992; Mauch *et al*., 1988; Schlumbaum *et al*., 1986). The expression of a number of chitinase genes appeared to be induced upon fungal infection (Majeau *et al*., 1990; Roby *et al*., 1990) and they were shown to accumulate around hyphal walls at infection sites in planta (Wubben *et al*., 1992). Several transgenic studies showed that by increasing the expression level of some chitinases the susceptibility of transformed plants to certain pathogens was significantly reduced (Broglie *et al*., 1991; Jach *et al*., 1995), providing an excellent tool for improving pest control. However, other studies were less conclusive. A 120-fold increase in expression of a tobacco class I chitinase did not result in any change in resistance to fungal infection (Neuhaus *et al*., 1991a). Similarly, down-regulation of the *Arabidopsis* ATHCHIA class III chitinase by antisense suppression did not increase susceptibility to fungi either (Samac *et al.*, 1994). Therefore it remains an open question whether the primary role of chitinases is plant defense or whether they have other functions.

There are several reports of developmentally-regulated chitinase expression, with specific isoforms being present only in certain organs and at specific stages, e.g. in flowers from tobacco (Neale *et al*., 1990; Trudel and Asselin, 1989), *Arabidopsis* (class IV AtEP3/AtchitIV; Passarinho *et al.* 2001 and class III ATHCHIA; Samac *et al.*, 1990), potato (SK2; Ficker *et al*., 1997), parsley (class II PcCHI1; Ponath *et al*., 2000) or rice (class I OsChia1; Takakura *et al*., 2000); in ripening banana fruit (Clendennen and May, 1997) or grape berries (class IV, VvChi4; Robinson *et al*., 1997); in roots from rice (class I RC24; Xu *et al*., 1996) or *Sesbania rostrata* (class III Srchi13; Goormachtig *et al*., 1998); in seeds of barley (class III Chi26; Leah *et al*., 1994), carrot (class IV EP3; van Hengel *et al*., 1998), pea (Chn; Petruzzelli *et al*., 1999), soybean (classIII; Yeboah *et al*., 1998) or in embryogenic cultures of carrot (class IV EP3; van Hengel *et al*., 1998), chicories (Helleboid *et al*., 2000), pine tree (Domon *et al.*, 2000), spruce (Dong and Dunstan, 1997; Egertsdotter, 1996). The specificity of expression of some chitinase genes suggests that they could also play a role in developmental processes such as pollination, senescence, root and root nodule development, seed germination and somatic embryogenesis. It was shown that chitinases could rescue the carrot somatic embryo mutant *ts11* (Baldan *et al*., 1997; de Jong *et al*., 1992; de Jong *et al*., 1993; Kragh *et al*., 1996) and could therefore play a crucial role in somatic embryo development. The study of Patil and Widholm (1997) also suggested the active participation of chitinases in development by over-expression of the maize Ch2 chitinase in tobacco that resulted in taller and stronger plants. Furthermore, the role of plant chitinases in Nod factor degradation during the formation of root nodules in the *Rhizobium*-legume symbiosis was shown in pea (Ovtsyna *et al*., 2000). Chitinase-mediated Nod factor degradation was already hypothesized several times and is especially interesting in line with the work of de Jong *et al*. (1993) showing that Nod factor-like molecules may exist in plants since rhizobial nodulation factors are also able to rescue the same carrot embryo mutant *ts11*.

In conclusion, chitinases are probably involved in a broad range of processes ranging from plant defense to development and there might be different functions associated with the different types of chitinases (reviewed in Graham and Sticklen, 1994). So far, attention has been mainly focused on agronomically important crops based on the preconceived idea that the natural role of plant chitinases is indeed in defense against pathogens. Very few studies were carried out in *Arabidopsis thaliana* and dealt with three different chitinases only (de A. Gerhardt *et al*., 1997; Passarinho *et al*., 2001; Samac *et al*., 1990; Verburg and Huynh, 1991). We have performed a survey of all putative chitinase genes in *Arabidopsis* and present here a detailed overview of their characteristics in relation with other plant chitinases. Based on these characteristics we discuss some of their possible functions and propose a modified annotation for some of the sequences, since in the release of the complete *Arabidopsis* genome sequence (The Arabidopsis Genome Initiative, 2000), most chitinases were annotated as “pathogen-induced or defense-related proteins”. In another database plant chitinases are annotated as being involved in the “biogenesis of cell wall”, based on homology with yeast chitinases. Moreover the AtEP3 endochitinase (Passarinho *et al.*, 2001) is classified as a protein involved in “cell rescue, defense, cell death and ageing – biogenesis of cell wall”; for sure a highly versatile protein.

## 2. Arabidopsis chitinase genes and their genomic distribution.

Using the word chitinase, we performed a keyword-based search on several *Arabidopsis* annotation databases (MATDB (MIPS (Munich Information Center for Protein Sequences) *Arabidopsis thaliana* DataBase); Mewes *et al*., 2000; http://mips.gsf.de/proj/thal/db/index.html), TIGR (The Institute for Genomic Research; http://www.tigr.org/tdb/e2k1/ath1/ath1.shtml) and D*At*A (Database of *Arabidopsis thaliana* Annotation; http://luggagefast.stanford.edu/group/arabprotein/index.html). Each search gave a slightly different result, mostly due to differences in clone names and annotations. We compared all returned accessions for redundancy and finally came to a total of 24 DNA sequences that, based on their annotation, encode putative chitinases (Table 1). The corresponding loci are distributed on all five chromosomes of the *Arabidopsis* genome (Figure 1), with a remarkable degree of clustering at the bottom of chromosome II where 6 putative genes are organized in tandem and in the middle of chromosome IV where 9 genes are organized in two clusters with 2 unrelated genes in between (Figure 1). It has now become obvious from several studies (Blanc *et al*., 2000; Vision *et al*., 2000) that the *Arabidopsis* genome contains large segmental duplications, suggesting that *Arabidopsis* could have originated from an ancient tetraploid ancestor (Blanc *et al*., 2000). It is likely that some of the duplicated genes have acquired a certain degree of specialization and are now expressed in different conditions. As found during systematic gene knockout in yeast (Ross-MacDonald *et al*., 1999), many insertion mutants in *Arabidopsis* do not show an obvious phenotype (Bouche and Bouchez, 2001; Pereira, 2000). This can be the result of gene redundancy or may point to a failure to detect subtle phenotypes perhaps only seen at the level of genome-wide gene expression as found in yeast (Beh *et al*., 2001).

Expressed Sequenced Tags (ESTs) were found for 16 of these sequences (Table 1) indicating that the corresponding genes are transcribed and most likely encode a functional protein, whereas the others are putative genes. This must be taken into consideration when drawing conclusions from their sequence, since they may be pseudogenes or are only expressed in conditions that were not studied in the various EST projects (Blanc *et al*., 2000).

## 3. Classification and structure of the Arabidopsis chitinase sequences.

The deduced amino acid sequences of all 24 accessions revealed that they all have a length of around 300 amino acids and a molecular weight of 25-35 kDa, which is typical for chitinases in general (Graham and Sticklen, 1994). The predicted proteins they encode belong to different groups according to the classification proposed for plant chitinases (Neuhaus *et al*., 1996). Based on their amino acid sequence all plant chitinases are endochitinases (EC 3.2.1.14) and have been organized in five different classes (Figure 2). Class I chitinases have a highly conserved N-terminal cysteine-rich region of approximately 40 amino acid residues that is involved in chitin-binding (Iseli *et al*., 1993). It is separated from the catalytic domain by a short proline-rich variable hinge region and the catalytic domain is often followed by a C-terminal extension that is involved in vacuolar targeting (Class Ia; Neuhaus *et al*., 1991b).

Class II chitinases lack both the N-terminal cystein-rich region and the C-terminal extension, but have a catalytic domain with a high sequence and structural similarity to that of class I chitinases. Class IV chitinases resemble class I chitinases with a very similar main structure, but they are significantly smaller due to four deletions distributed along the chitin-binding domain and the catalytic region. Class III chitinasesare more similar to fungal and bacterial chitinases than to other plant chitinases (Graham and Sticklen, 1994), except for class V chitinases, that also belong to the family 18 of glycosyl hydrolases whereas all other classes belong to family 19. In addition, class V chitinases have a C-terminal extension for vacuolar targeting and may contain a chitin-binding domain as well (Heitz *et al*., 1994; Ponstein *et al*., 1994). Finally, cass III and class V chitinases display an additional lysozymal activity (Heitz *et al*., 1994; Majeau *et al*., 1990).

As in all plants analyzed to date (Graham and Sticklen, 1994), members of all five classes are present in the *Arabidopsis* genome. It is also remarkable that classes I and III are poorly represented with only one member each (Figure 2), whereas the other classes are more abundant, especially classes IV and V with 9 members each. It is also noteworthy that the class I chitinase contains a C-terminal extension, hence belongs to subclass Ia, and none of he class V members possesses a chitin-binding domain.

Figure 3 shows the phylogenetic tree generated with the 24 sequences by using the CLUSTALW Multiple Sequence Alignment program at the GenomeNet WWW server (http://clustalw.genome.ad.jp/). The different classes are nicely clustered and it is clear that class V has diverged from the other classes very early during evolution. It also seems that the very similar classes I and IV may have arisen from class II in which they are imbedded. Araki and

**Table 1. *Arabidopsis* chitinase annotations**

| Locus (Clone name) | Chr | Accessions | Annotation | Length (aa) | MW (kDa) | No of ESTs found | Proposed function | Class |
|---|---|---|---|---|---|---|---|---|
| At1g02360 (T6A9.15) | I | AAG00887.1 gi9857532 | Putative endochitinase | 272 | 30.1 | 4 | Biogenesis of cell wall (MATDB) | II |
| At1g05870 (T20M3.10) | I | AAF29390.1 gi6850313 | Putative class I chitinase | 321 | 35.6 | >8 | - | II |
| At1g56680 (F25P12.88) | I | AAG09096.1 gi9954745 | Putative chitinase | 280 | 31.2 | - | Pathogen (fungi) response (TIGR). Biogenesis of cell wall (MATDB) | IV |
| At2g43570 (F18O19.32) | II | AAB64049 gi2281113 | Putative endochitinase | 277 | 29.8 | 3 | Pathogen (fungi) response (TIGR). Biogenesis of cell wall (MATDB) | IV |
| At2g43580 (F18O19.31) | II | AAB64048 gi2281112 | Putative endochitinase | 265 | 28.8 | - | Pathogen (fungi) response (TIGR). Biogenesis of cell wall (MATDB) | IV |
| At2g43590 (F18O19.30) | II | AAB64047 gi2281111 | Putative endochitinase | 264 | 28.4 | 7 | Pathogen (fungi) response (TIGR). Biogenesis of cell wall (MATDB) | IV |
| At2g43600 (F18O19.29) | II | AAB64046 gi2281110 | Putative endochitinase | 273 | 30.9 | - | Pathogen (fungi) response (TIGR). Biogenesis of cell wall (MATDB) | IV |
| At2g43610 (F18O19.28) | II | AAB64045 gi2281109 | Putative endochitinase | 281 | 30 | 6 | Pathogen (fungi) response (TIGR). Biogenesis of cell wall (MATDB) | IV |
| At2g43620 (F18O19.27) | II | AAB64044 gi2281108 | Putative endochitinase | 283 | 30.4 | 8 | Pathogen (fungi) response (TIGR). Biogenesis of cell wall (MATDB) | IV |
| At3g12500 (T2E22.18) | III | AAG51023.1 gi12321966 | Basic chitinase | 335 | 36.2 | 9 | Pathogen-induced-Defense related protein | I |
| At3g16920 (K14A17.4) | III | BAA94976.1 gi7670022 | Putative basic chitinase | 333 | 36.7 | 8 | Biogenesis of cell wall (MATDB) | II |
| At3g147540 (F1P2.90) | III | CAB61980 gi6522537 | Endochitinase - like protein | 214 | 23.3 | - | Cell rescue, defense, cell death and aging - biogenesis of cell wall (MATDB) | IV |
| At3g54420 (T12E18.110) | III | CAB81807 gi7288020 | Class IV chitinase | 273 | 29.4 | 4 | Cell rescue, defense, cell death and aging - biogenesis of cell wall (MATDB) | IV |
| At4g01700 (T15B16.5) | IV | AAC72865 gi3855959 | Putative chitinase | 280 | 31.5 | 10 | Biogenesis of cell wall (MATDB) | II |

*(continues)*

**Table 1. *Arabidopsis* chitinase annotations** (continued)

| Locus (Clone name) | Chr | Accessions | Annotation | Length (aa) | MW (kDa) | EST s | Proposed function | Class |
|---|---|---|---|---|---|---|---|---|
| At4g19720 (T16H5.80) | IV | CAA19692.1 gi3250684 | Chitinase -like protein (TIGR) Similar to tobacco chitinase/lysozyme PZ precursor (MATDB) | 421 | 46.9 | 3 | Pathogen-induced- Defense related protein | V |
| At4g19730 (T16H5.90) | IV | CAB78975.1 gi7268769 | Chitinase -like protein (TIGR) Similar to tobacco chitinase/lysozyme PZ precursor (MATDB) | 332 | 36.7 | 2 | Pathogen-induced- Defense related protein | V |
| At4g19740 (T16H5.100) | IV | CAB78976.1 gi7268770 | Chitinase -like protein (TIGR) Similar to tobacco chitinase/lysozyme PZ precursor (MATDB) | 272 | 30.5 | - | Pathogen-induced- Defense related protein | V |
| At4g19750 (T16H5.110) | IV | CAB78977.1 gi7268771 | Chitinase -like protein (TIGR) Similar to tobacco chitinase/lysozyme PZ precursor (MATDB) | 371 | 40.4 | 2 | Pathogen-induced- Defense related protein | V |
| At4g19760 (T16H5.120) | IV | CAB78978.1 gi7268772 | Chitinase -like protein (TIGR) Similar to tobacco chitinase/lysozyme PZ precursor (MATDB) | 365 | 40.1 | 2 | Pathogen-induced- Defense related protein | V |
| At4g19770 (T16H5.130) | IV | CAB78979.1 gi7268773 | Chitinase -like protein (TIGR) Similar to tobacco chitinase/lysozyme PZ precursor (MATDB) | 248 | 27.4 | - | Pathogen-induced- Defense related protein | V |
| At4g19800 (T16H5.160) | IV | CAB78982.1 gi7268776 | Chitinase -like protein (TIGR) Similar to tobacco chitinase/lysozyme PZ precursor (MATDB) | 398 | 44.4 | - | Pathogen-induced- Defense related protein | V |
| At4g19810 (T16H5.170) | IV | CAB78983.1 gi7268777 | Chitinase -like protein (TIGR) Similar to tobacco chitinase/lysozyme PZ precursor (MATDB) | 379 | 41.1 | 5 | Pathogen-induced- Defense related protein | V |
| At4g19820 (T16H5.180) | IV | CAB78984.1 gi7268778 | Chitinase -like protein (TIGR) Similar to tobacco chitinase/lysozyme PZ precursor (MATDB) | 366 | 40.9 | - | Pathogen-induced- Defense related protein | V |
| At5g24090 (MZF18.2) | V | BAA21861.1 gi2342435 | Acidic endochitinase | 302 | 33.1 | 3 | C-compound and carbohydrate utilization, cytokinesis and extracellular/secretion protein [(1)] | III |

All non-redundant sequences annotated as chitinase in the various *Arabidopsis* databases are indicated here, with the corresponding locus and clone names, as well as the protein accession numbers and the exact annotation from the database, which name is indicated when the annotations differed from one another. The length and the molecular weight (MW) of each predicted amino acid sequence is also shown, as well as the number of ESTs found for each one of them. The second to last column shows the automatically derived functions proposed in the MATDB and TIGR databases. The annotation marked [(1)] is based on sequence homology with a yeast endochitinase involved in polarized cell growth and cell separation (Kuranda and Robbins, 1991). In ÒPZ-precursorÓ (second part of the table), PZ stands for PR-protein isolated by zinc chelate chromatography (Heitz *et al*., 1994). The last column contains the putative class to which the chitinase genes belong, as we determined based on their sequence and added to the original annotation.

Torikata (1995) have indeed suggested that class I chitinases arose from class II chitinases by insertion of the chitin-binding domain. This probably occurred in the case of class IV chitinases as well, considering their degree of similarity with class I members, including the presence of the chitin-binding domain.

## 4. Sequence characteristics of the Arabidopsis chitinases.

Based on the classes obtained from the phylogenetic tree, the deduced amino acid sequences of all chitinase genes were compared to each other by multiple sequence alignment and the presence of elements essential for chitinase activity was analyzed for each sequence.

Figure 4 shows the sequences of class I and class III chitinases, both of which represent actual genes that were isolated by Samac *et al*. (1990). The class I chitinase sequence contains all characteristics of class I chitinases including the C-terminal extension, specific of subclass Ia, indicating that it is targeted to the vacuole. All residues shown to be involved in substrate binding and catalytic activity are also present (Garcia-Casado *et al*., 1998) and indicate that it is most likely an active chitinase and one of that is actively transcribed (Samac *et al*., 1990). The same holds true for the class III chitinase, of which the catalytic domain possesses all essential residues known to date (Watanabe *et al*., 1993).

Figure 5 shows the multiple alignment of the class II chitinase sequences and one can see that they share a relatively high degree of similarity, especially in the catalytic domain. However it also appears that two of these sequences do not possess all conserved residues essential for chitinase activity. As a matter of fact, only the sequences of the two underlined accessions fulfill all requirements described by Garcia-Casado *et al*. (1998). For example, the H-E-T-T motif including the essential glutamic acid residue shown in bold is absent from the two other sequences. The same holds true for the first cysteine in the Chitinase 19_1 conserved domain as well as for most of the residues in bold that are essential for catalytic activity and the boxed residues involved in substrate binding. Nevertheless these residues were only shown to play a specific role in a class I chitinase (Garcia-Casado *et al*., 1998) and there are no reports so far of a similar study with class II chitinases. Therefore it could still be that especially the residues involved in substrate binding (boxed) are different in this class. We can eliminate the last 2 sequences (At1g05870 and At3g16920) as non-active chitinases based on the absence of the H-E-T-T motif and of some of the other residues essential for catalytic activity. Furthermore, At1g05870 and At3g16920 were also put together at the bottom of the phylogenetic tree (Figure 3) indicating that although they are similar to each other they also diverge considerably from the other class II members. Interestingly the sequences At1g02360 and At4g01700 considered as encoding active chitinases are also paired in the dendrogram shown in Figure 3 and are located on chromosomal regions that were shown to be duplicated (i.e the top of chromosome I and the top of chromosome IV; Blanc *et al*., 2000) and are therefore likely to represent a duplication of the same gene.

Figure 6 shows the same comparison for class IV chitinases to which the only other *Arabidopsis* chitinase studied, AtEP3/AtChitIV (At3g54420; de A. Gerhardt *et al*., 1997; Passarinho *et al*., 2001) belongs. In this class the degree of conservation is very high and all elements specific for class IV chitinases are present, except for accession At3g47540 that lacks the chitin-binding domain as well as the accompanying hinge region. Nevertheless it was put in class IV, since its shorter catalytic domain is more closely related to that of this class than to that of class II chitinases. It is also shorter than the other class IV chitinase genes in the second half of the catalytic domain where it also lacks some of the important amino acid residues (i.e. glutamate-170 and serine-172, as seen in the At2g43590 sequence). Furthermore, there was no EST found for At3g47540, so it could very well be that it represents a pseudogene. There were three other sequences for which no EST was found (marked by the asterisk) and those also appear to lack some essential amino acids in the second half of the catalytic domain, especially At2g43600 that lacks the essential glutamic acid residue at position 140 and is therefore probably not active as a chitinase. It is also remarkable that in this class some of the residues shown to be involved in substrate binding in class I chitinases are here consistently different (Garcia-Casado *et al*., 1998). For example the H-E-T-T motif seems to be replaced by H-E-[TS]-G, and the tryptophan residue that should have been at position 153 (see the At2g43590 sequence) is replaced by a tyrosine. The same holds true for the glutamine-212 and the lysine-214 of the same sequence that are replaced by a valine. These differences most likely reflect a class-related difference in substrate specificity, which is also illustrated by the tyrosine (shown by the arrow) that was shown to be essential for substrate binding, but not for catalysis in the class I chitinase (Verburg *et al*., 1993) and is replaced by a phenylalanine, especially in sequence At3g54420 (i.e. AtEP3/AtChitIV), of which we know that it is an active chitinase (Passarinho *et al*., 2001). As for class II chitinases, based on the missing essential amino acid residues and the failure to find ESTs we can con-

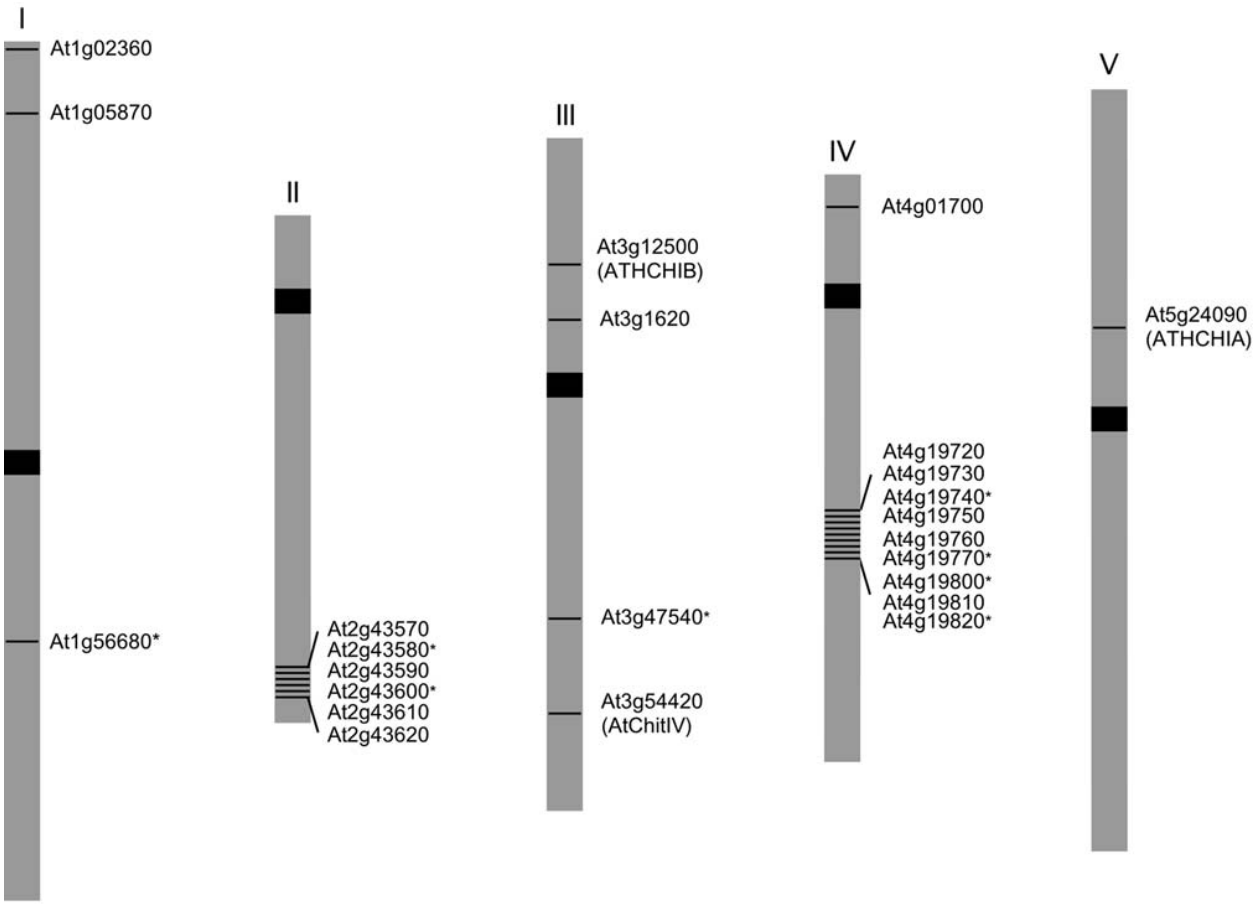



**Figure 1. Genomic distribution of the Arabidopsis chitinase-encoding genes.**
The locus of each accession is shown on the individual chromosomes. The (*) marks the putative genes, for which no ESTs were found.

clude that the accessions At1g56680, At2g43580, At2g43600 and At3g47540 are not very likely to encode active chitinases. It is also noteworthy that the majority of class IV chitinases is clustered at the bottom of chromosome II and is also found on the lower arm of chromosome III (Figure 1) that also seems to be an area duplicated on chromosome II (Blanc *et al*., 2000).

Figure 7 presents the multiple alignment of class V chitinases. The chitinases of this class are longer than the members of the other classes. They also seem to possess additional motifs, which were not found in other classes and of which we do not know the functional relevance. Little is known about class V chitinases and we can therefore only base our analysis on what is known for the glycosyl hydrolase family 18 (Watanabe *et al*., 1993), of which the conserved characteristic motif represents a small segment of the whole protein. In this small conserved region we can already see that two members of this class (At4g19720 and At4g19820) deviate from the others since a lysine residue (arrow) replaces the proposed essential glutamic acid. This resembles the situation of concanavalin B present in seeds of *Canavalia ensiformis* (Hennig *et al*., 1995), where the glutamic acid residue is replaced by a glutamine. As a consequence, concanavalin B, a close relative of family 18 chitinases, lost its enzymatic activity, but retained its carbohydrate-binding function (Hennig *et al*., 1995).

Concanavilin B is biochemically and structurally similar to narbonin that is a storage protein found in seeds of *Vicia narbonensis* (Hennig *et al*., 1992; Nong *et al*., 1995) and could be involved in “trapping” carbohydrate molecules necessary for the seed. A similar function could be proposed here for At4g19820 and At4g19720. The other sequences, including those for which no EST was found,

all have an intact catalytic site and should therefore be active class V chitinases. As seen for class IV chitinases they are also clustered on a particular chromosomal location, on the lower arm of chromosome IV (Figure 1), but this region does not seem to have been duplicated elsewhere in the genome.

## 5. Putative function and reannotation of the Arabidopsis chitinase sequences.

In order to obtain additional clues with respect to the putative function of all chitinases, each sequence was also analyzed for the presence of additional specific motifs by using the InterPro domain search (http://www.ebi.ac.uk/interpro/; Apweiler *et al*., 2001) and for the presence of targeting sequences using the PSORT (http://psort.nibb.ac.jp/) and targetP (http://www.cbs.dtu.dk/services/TargetP/; Emanuelsson *et al*., 2000) servers. A PSI-BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/; Altschul *et al*., 1997) was also performed in order to obtain more functional data on similar chitinases. The results of this analysis are detailed in Table 2.

### *5.1. Class I*

In *Arabidopsis thaliana*, class I chitinases are represented by one member only, ATHCHIB (At3g12500) that was also the first chitinase gene isolated in *Arabidopsis* (Samac *et al*., 1990). It is a basic chitinase and is most likely target-

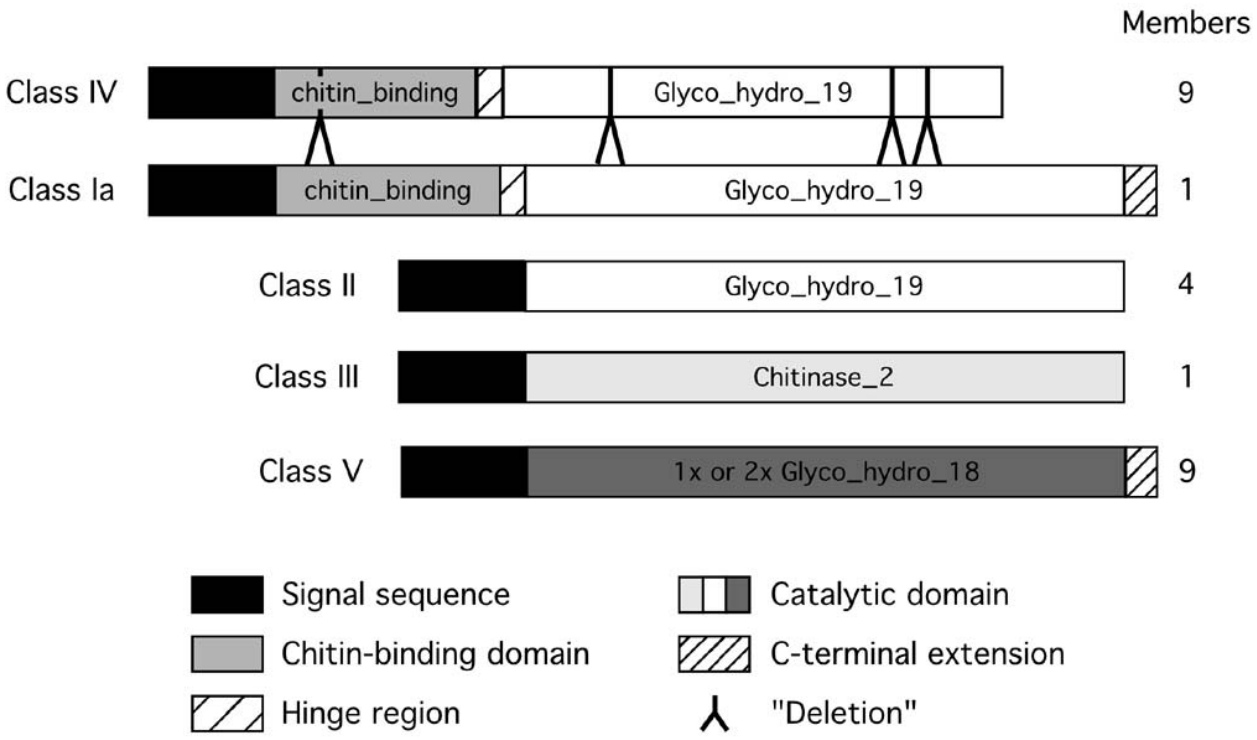



**Figure 2. Classification and structure of the chitinase proteins found in the Arabidopsis genome.**
The structural domains are schematically represented and include the names of the corresponding signatures found in the Pfam protein families database (Bateman *et al*., 2000). Chitin_binding corresponds to pfam00187 (chitin binding, recognition protein); Glyco_hydro_19 to pfam 00182 (chitinases, class I, i.e. family 19); glyco_hydro_18 (i.e. family 18) to pfam00704 and chitinase_2 to pfam 00192 (chitinases, family 2) that is a subset of family 18. The numbers of members present in each class are indicated on the right.(Adapted from Collinge *et al*., 1993).

**Table 2. Characteristics and reannotation of the *Arabidopsis* chitinase genes.**

| Locus | InterPro domain search | Targeting | Similarities | Reannotation - Remarks |
|---|---|---|---|---|
| At1g02360 | IPR000726 Chitinase family 19 (pfam00182 Glyco_hydro_19 plus PS00773 CHITINASE 19_1 and PS00774 CHITINASE 19_2) | PSORT: outside TargetP: secretory pathway, probable signal sequence 32 | At4g01700; chitinase precursors AAD54936.1/ AAD54935.1 from *Petroselinum crispum* cultured cells; class II chitinase CAA57773.1 from *Arachis hypogea* (Chi2;1 induced by fungal spores; (Kellmann *et al*., 1996)); class II chitinase AAF00131.1 from *Fragaria x ananassa*; etcÉ | Transcribed sequence encoding a most likely active secreted class II chitinase, possibly involved in pathogen responses. |
| At1g05870 | IPR000726 Chitinase family 19 (pfam00182 Glyco_hydro_19) | PSORT: to the ER (membrane) | basic chitinase BAA94976.1 from *A. thaliana* (At3g19620); basic chitinase CAA78843.1 from *Lycopersicon esculentum* (induced by *C. fulvum*, (Danhash *et al*., 1993)); class I chitinases from *Arabis* (Bishop *et al*., 2000); etcÉ | Transcribed sequence similar to a class II chitinase, but is probably inactive as a chitinase. Unknown function. |
| At1g56680* | IPR001002 Chitin-binding domain (pfam00187 chitin_binding)<br>IPR000726 Chitinase family 19 (pfam00182 Glyco_hydro_19)<br>IPR000531 | PSORT: outside TargetP: secretory pathway | class IV chitinase precursor AAB01665.1 from *Brassica napus* (induced by SA, leaf senescence; (Hanfrey *et al*., 1996)); basic endochitinase CH4B CAA43708 from *B. napus* (induced by *Phoma lingam*; (Rasmussen *et al*., 1992)); putative endochitinase AAB6404.1 from *A. thaliana* (At2g43590); etcÉ | Putative gene (no ESTs found) encoding a protein similar to a probably inactive class IV chitinase. |
| At2g43570 | IPR001002 Chitin-binding domain (pfam00187 chitin_binding)<br>IPR000726 Chitinase family 19 (pfam00182Glyco_hydro_19 and PS00773 CHITINASE 19_1) | PSORT: outside (0.82) or vacuole (0.43) TargetP: secretory pathway, probable signal sequence 24 | same as At1g58860<br>plus seed chitinase A PIR: P29022 from *Zea mays* (antifungal role; (Huynh *et al*., 1992)); etcÉ | Transcribed sequence encoding a most likely active secreted class IV chitinase, possibly involved in pathogen responses and development. |
| At2g43580* | IPR001002 Chitin-binding domain (pfam00187 chitin_binding and PS00026 CHITIN_BINDING)<br>IPR000726 Chitinase family 19 (pfam00182 Glyco_hydro_19; PS00773 CHITINASE 19_1 and PS00774 CHITINASE 19_2)<br>IPR001687 ATP/GTP-binding site motif A (P-loop) (PS00017 ATP_GTP_A) | PSORT: outside TargetP: secretory pathway, probable signal sequence 24 | basic endochitinase CH4B CAA43708 from *B. napus* (induced by *P. lingam*; (Rasmussen *et al*., 1992)); At2g43590; class IV endochitinase AtChitIV CAA74930.1 from *A. thaliana* (de A. Gerhardt *et al*., 1997; Passarinho *et al*., 2001); class IV chitinase CAA40474.1 from *Phaseolus vulgaris* (induced by *Fusarium solani*; (Lange *et al*., 1996)); etcÉ | Putative gene (no EST found) encoding a probably inactive secreted class IV chitinase. Unknown function. |
| At2g43590 | IPR000726 Chitinase family 19 (pfam00182 Glyco_hydro_19; PS00773 CHITINASE 19_1 and PS00774 CHITINASE 19_2)<br>IPR001002 Chitin-binding domain (pfam00187 chitin_binding and PS00026 CHITIN_BINDING) | PSORT: outside TargetP: secretory pathway, probable signal sequence 22 | basic endochitinase CH4B CAA43708 from *B. napus* (induced by *P. lingam*; (Rasmussen *et al*., 1992)); At2g43580; class IV endochitinase AtChitIV CAA74930.1 from *A. thaliana* (de A. Gerhardt *et al*., 1997; Passarinho *et al*., 2001); class IV endochitinase AAB65776.1 from *Vitis vinifera* (expressed in flowers and berries, highly induced in ripening berries; (Robinson *et al*., 1997)); etcÉ | Transcribed sequence encoding a most likely active secreted class IV chitinase, possibly involved in pathogen responses and development. |
| At2g43600* | IPR000726 Chitinase family 19 (pfam00182 Glyco_hydro_19; PS00773 CHITINASE 19_1 and PS00774 CHITINASE 19_2)<br>IPR001002 Chitin-binding domain (pfam00187 chitin_binding and PS00026 CHITIN_BINDING) | PSORT: vacuole (0.88), outside (0.82) TargetP: secretory pathway, probable signal sequence 28 | At1g56680; At2g43610 & At2g43620; basic endochitinase CH4B CAA43708 from *B. napus* (induced by *P. lingam*; (Rasmussen *et al*., 1992)); At2g43580 & At2g43590; chitinase BAA22965.1 from *Chenopodium amaranticolor*; etcÉ | Putative gene (no EST found) encoding a probably inactive secreted class IV chitinase. Unknown function. |

The results of the InterPro domain search are presented here as well as the highest PSORT scores (numbers in between brackets) together with the results of the TargetP search. The number following Òprobable signal sequenceÓ in the TargetP results is the proposed length of this sequence in amino acids. The fourth column contains the highest scores obtained when performing a PSI-BLAST search (Altschul *et al*., 1997) with the individual deduced protein sequences. These are in the same order as the results of this search, i.e. in decreasing degree of similarity. Bibliographical references, when available, were included as well as some additional information. The last column is a synthesis of these data combined with the data presented in the previous sections.

*(continues)*

**Table 2. Characteristics and reannotation of the *Arabidopsis* chitinase genes (continued).**

| Locus | InterPro domain search | Targeting | Similarities | Reannotation - Remarks |
|---|---|---|---|---|
| At2g43610 | IPR000726 Chitinase family 19 (pfam00182 Glyco_hydro_19)<br>IPR001002 Chitin-binding domain (pfam00187 chitin_binding and PS00026 CHITIN_BINDING) | PSORT: outside<br>TargetP: secretory pathway, probable signal sequence 28 | At2g43620; At1g56680; At2g43600; basic endochitinase CH4B CAA43708 from *B. napus* (induced by *P. lingam*; (Rasmussen *et al*., 1992)); At2g43590; chitinase BAA22968.1 from *C. amaranticolor*; etcÉ | Transcribed sequence encoding a most likely active secreted class IV chitinase, possibly involved in pathogen responses and development. |
| At2g43620 | IPR000726 Chitinase family 19 (pfam00182 Glyco_hydro_19)<br>IPR001002 Chitin-binding domain (pfam00187 chitin_binding and PS00026 CHITIN_BINDING) | PSORT: outside<br>TargetP: secretory pathway, probable signal sequence 21 | At2g43610; At1g56680; At2g43600; basic endochitinase CH4B CAA43708 from *B. napus* (induced by *P. lingam*; (Rasmussen *et al*., 1992)); At2g43590; chitinase BAA22968.1 from *C. amaranticolor*; etcÉ | Transcribed sequence encoding a most likely active secreted class IV chitinase, possibly involved in pathogen responses and development. |
| At3g12500 | IPR000726 Chitinase family 19 (pfam00182 Glyco_hydro_19; PS00773 CHITINASE 19_1 and PS00774 CHITINASE 19_2)<br>IPR001002 Chitin-binding domain (pfam00187 chitin_binding and PS00026 CHITIN_BINDING) | PSORT: outside<br>TargetP: secretory pathway, probable signal sequence 32 | is class I chitinase ATHCHIB from *A. thaliana* (AAA32769; (Samac *et al*., 1990)); class I chitinases from *Arabis* (Bishop *et al*., 2000); endochitinase CH25 precursor PIR: Q09023 from *B. napus* (Hamel and Bellemare, 1993); endochitinase precursor AAA34070.1 from *N. tabacum* (inhibition in cell cultures by auxin and cytokinin; (Shinshi *et al*., 1987)); etcÉ | Transcribed sequence encoding a vacuolar active class I chitinase (not compatible with computer-proposed targeting).<br>Developmentally regulated, possibly involved in pathogen responses and senescence, linked to ethylene signaling. |
| At3g16920 | IPR000726 Chitinase family 19 (pfam00182 Glyco_hydro_19) | PSORT: outside<br>TargetP: secretory pathway, probable signal sequence 23 | At1g05870; class I chitinases from *Arabis* (Bishop *et al*., 2000); class II chitinase S26625 from *Solanum tuberosum* (Wemmer *et al*., 1994); basic class Ia chitinase CAA78843.1 from *L. esculentum* (induced by *C. fulvum*, (Danhash *et al*., 1993)); etcÉ | Transcribed sequence similar to a class II chitinase, but is probably inactive as a chitinase. Possible defense function. |
| At3g47540* | IPR000726 Chitinase family 19 (pfam00182 Glyco_hydro_19) | PSORT: outside<br>TargetP: secretory pathway, probable signal sequence 34 | At2g43590; basic endochitinase CH4B CAA43708 from *B. napus* (induced by *P. lingam*; (Rasmussen *et al*., 1992)); At2g43580; class IV endochitinase AtChitIV CAA74930.1 from *A. thaliana* (de A. Gerhardt *et al*., 1997; Passarinho *et al*., 2001); etcÉ | Putative gene (no EST found) encoding an inactive secreted class IV chitinase, possibly involved in pathogen responses. |
| At3g54420 | IPR000726 Chitinase family 19 (pfam00182 Glyco_hydro_19; PS00773 CHITINASE 19_1)<br>IPR001002 Chitin-binding domain (pfam00187 chitin_binding and PS00026 CHITIN_BINDING) | PSORT: outside<br>TargetP: secretory pathway, probable signal sequence 27 | is class IV endochitinase AtChitIV CAA74930.1 from *A. thaliana* (de A. Gerhardt *et al*., 1997; Passarinho *et al*., 2001); class IV chitinase CAA40474.1 from *P. vulgaris* (induced by *F. solani*; (Lange *et al*., 1996)); class IV endochitinase AAB65776.1 from *V. vinifera* (expressed in flowers and berries, highly induced in ripening berries; (Robinson *et al*., 1997)); basic endochitinase CH4B CAA43708 from *B. napus* (induced by *P. lingam*; (Rasmussen *et al*., 1992)); etcÉ | Transcribed sequence encoding an active secreted class IV chitinase, possibly involved in development and pathogen responses. |
| At4g01700 | IPR000726 Chitinase family 19 (pfam00182 Glyco_hydro_19 and PS00774 CHITINASE 19_2) | PSORT: ER<br>TargetP: secretory pathway, probable signal sequence 30 | At1g02360; class II chitinase CAA57773.1 from *A. hypogea* (Chi2;1 induced by fungal spores; (Kellmann *et al*., 1996); chitinase precursors AAD54936.1/AAD54935.1 from *P. crispum* cultured cells; etcÉ | Transcribed sequence encoding a most likely active secreted class II chitinase, possibly involved in pathogen responses. |
| At4g19720 | IPR001233 Glycosyl hydrolases family 18 (2x) (2x pfam00704, Glyco_hydro_18)<br>IPR000531 TonB-dependent receptor protein (PS00430 TONB-DEPENDENT_REC1)<br>IPR000677 2-S Globulin family (pfam02220 Narbonin) | PSORT: peroxisome (0.6) or cytoplasm (0.4) | All At4g19xx members; Class V chitinase CAA54373 from *N. tabacum* (stress-induced; (Melchers *et al*., 1994)); chitinase/lysozyme PZ precursor PIR: S51591 from *N. tabacum* (expressed in healthy tissues, (Heitz *et al*., 1994)); receptor like kinase CHRK1 AAD52097 from *N. tabacum* (pathogen-induced; (Kim *et al*., 2000)); etcÉ | Transcribed sequence encoding a probably inactive cellular class V chitinase, like concanavalin B. Possibly involved in perception and/or recruitment of chitin-derived molecules during pathogen responses. |

*(continues)*

**Table 2. Characteristics and reannotation of the *Arabidopsis* chitinase genes (continued).**

| Locus | InterPro domain search | Targeting | Similarities | Reannotation - Remarks |
|---|---|---|---|---|
| At4g19730 | IPR001233 Glycosyl hydrolases family 18 (pfam00704, Glyco_hydro_18) | PSORT: peroxisome (0.6) or cytoplasm (0.4) | Like At4g19720 | Transcribed sequence encoding a most likely active cellular class V chitinase, possibly involved in pathogen responses. |
| At4g19740 | IPR001233 Glycosyl hydrolases family 18 (pfam00704, Glyco_hydro_18) | PSORT: peroxisome (0.6) or cytoplasm (0.4) | Like At4g19720 | Putative gene (no EST found) encoding a most likely active cellular class V chitinase. Unknown function. |
| At4g19750 | IPR001233 Glycosyl hydrolases family 18 (pfam00704, Glyco_hydro_18)<br>IPR001064 Crystallin (PS00225 CRYSTALLIN_BETAGAMMA)<br>IPR001472 Bipartite nuclear localization signal (PS50079 NLS_BP) | PSORT: nucleus (0.76) or peroxisome (0.75) | Like At4g19720 | Transcribed sequence encoding a most likely active cellular class V chitinase, possibly involved in pathogen responses. |
| At4g19760 | IPR001233 Glycosyl hydrolases family 18 (pfam00704, Glyco_hydro_18)<br>IPR001064 Crystallin (PS00225 CRYSTALLIN_BETAGAMMA)<br>IPR001472 Bipartite nuclear localization signal (PS50079 NLS_BP) | PSORT: nucleus (0.76) or peroxisome (0.75) | Like At4g19720 | Transcribed sequence encoding a most likely active cellular class V chitinase, possibly involved in pathogen responses. |
| At4g19770 | IPR001233 Glycosyl hydrolases family 18 (2x) (2x pfam00704, Glyco_hydro_18)<br>IPR001579 Chitinases family 18 and 2 (PS01095 CHITINASE_18)<br>IPR001064 Crystallin (PS00225 CRYSTALLIN_BETAGAMMA) | PSORT: outside (0.37), vacuole (0.32) or peroxisome (0.28) | Like At4g19720 | Putative gene (no EST found) encoding a most likely active cellular class V chitinase. Unknown function. |
| At4g19800 | IPR001233 Glycosyl hydrolases family 18 (pfam00704, Glyco_hydro_18)<br>IPR001064 Crystallin (PS00225 CRYSTALLIN_BETAGAMMA) | PSORT: peroxisome (0.6) or cytoplasm (0.4) | Like At4g19720 | Putative gene (no EST found) encoding a most likely active cellular class V chitinase. Unknown function. |
| At4g19810 | IPR001233 Glycosyl hydrolases family 18 (pfam00704, Glyco_hydro_18) | PSORT: outside<br>TargetP: secretory pathway, probable signal sequence 29 | Like At4g19720 | Transcribed sequence encoding a most likely active secreted class V chitinase, possibly involved in pathogen responses. |
| At4g19820 | IPR001233 Glycosyl hydrolases family 18 (pfam00704, Glyco_hydro_18) | PSORT: outside (0.5) or peroxisome (0.3)<br>TargetP: secretory pathway, probable signal sequence 20 | Like At4g19720 | Putative gene (no EST found) encoding a probably inactive secreted class V chitinase, like concanavalin B. Unknown function. |
| At5g24090 | IPR001579 Chitinases family 18 and 2 (pfam00192 chitinase_2 and PS01095 CHITINASE_18) | PSORT: outside<br>TargetP: secretory pathway, probable signal sequence 21 | is acidic endochitinase ATHCHIA from *A. thaliana* (BAA21861.1; (Samac *et al.*, 1990)); ChiA locus (Kawabe *et al.*, 1997); Hevamine A PIR:P23472 from *Hevea brasiliensis* (putative role in cessation of latex flow; (Jekel *et al.*, 1991)); Acidic endochitinase precursor PIR:P51614 from *V. vinifera* (pathogen-induced, putative role in systemic acquired resistance; (Busam *et al.*, 1997)); etcÉ | Transcribed sequence encoding an active secreted class III chitinase, possibly involved in response to specific pathogens. |

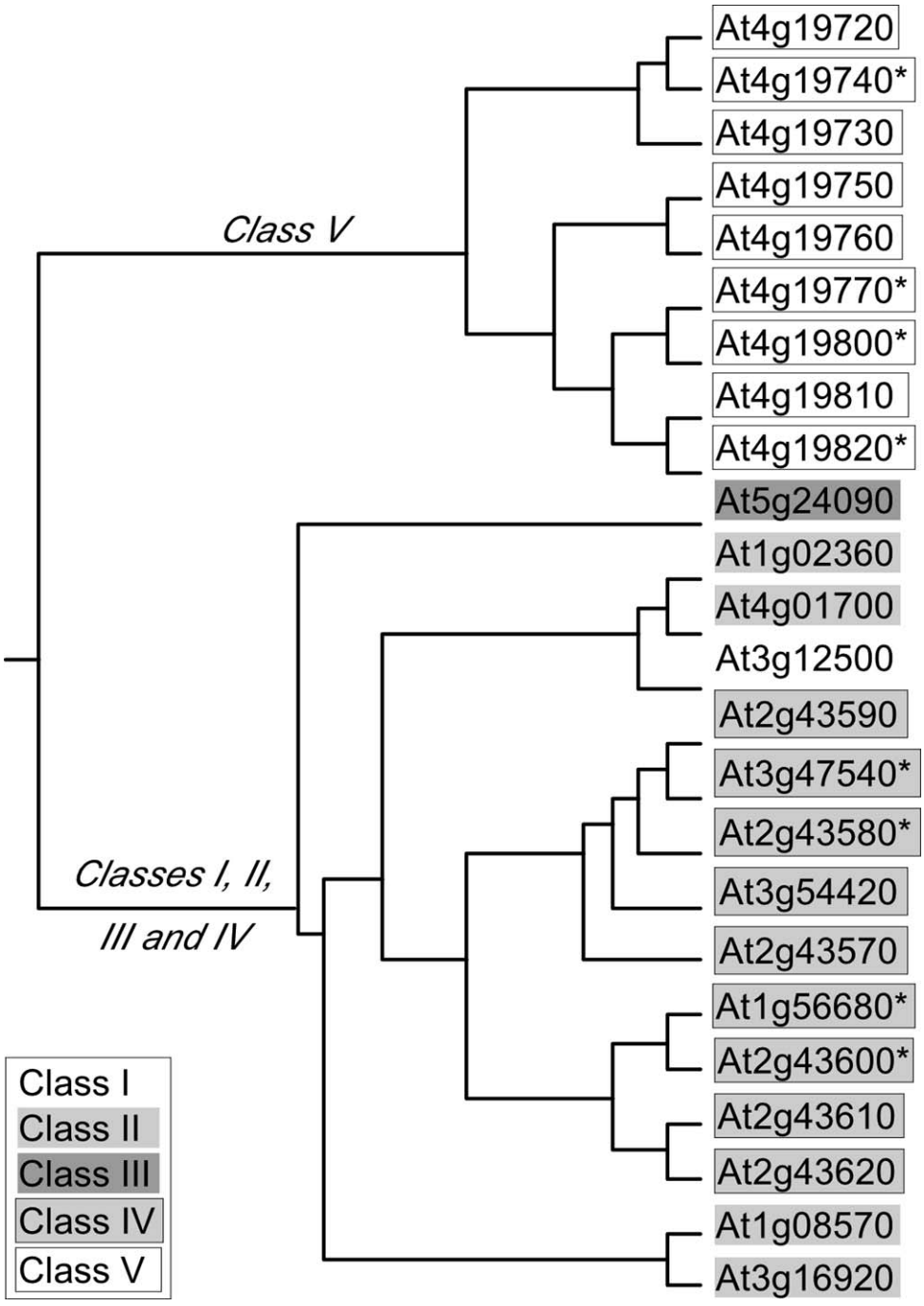
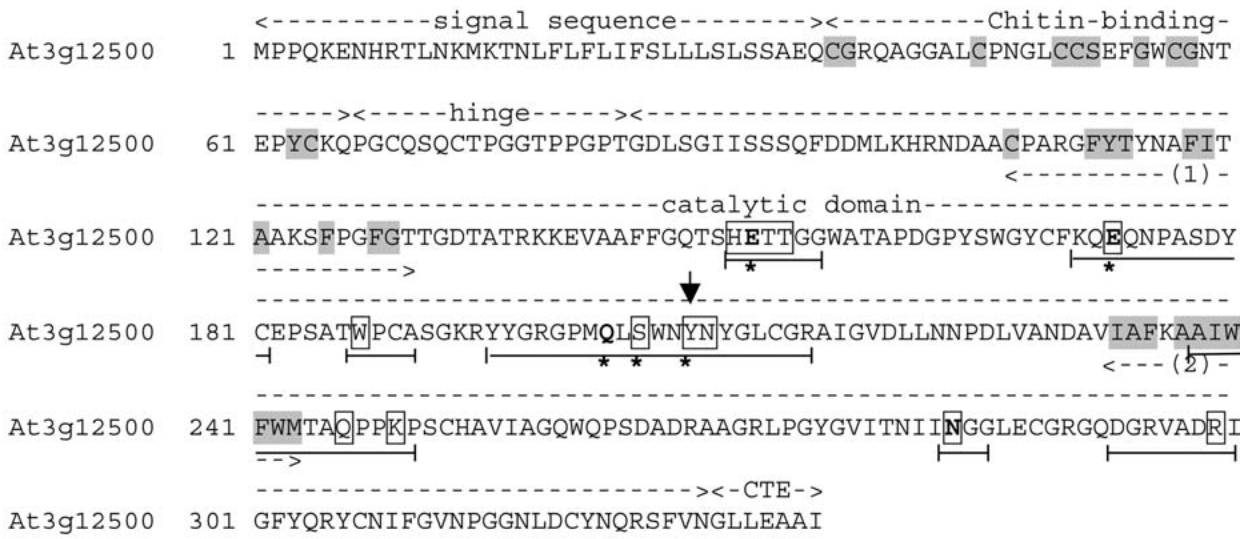
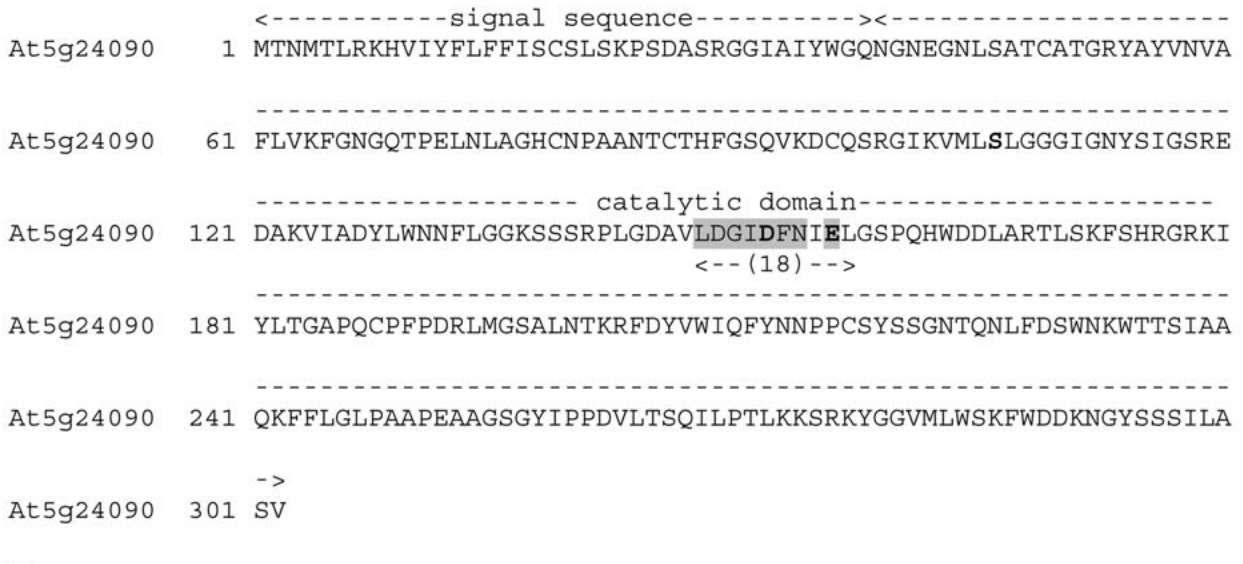



**Figure 3. Phylogenetic tree of the Arabidopsis chitinase proteins.**
The dendrogram was generated by using the CLUSTALW Multiple Sequence Alignment program at the GenomeNet WWW server (http://clustalw.genome.ad.jp/). The belonging classes of each accession are indicated by the shading and boxes around their names and as in all figures the (*) marks the putative genes, for which no ESTs were found.




**A.**




**B.**

**Figure 4. Sequences and structural features of the Arabidopsis class I and class III chitinases.**
Structural domains as described in Figure 2 are indicated above the sequences. PROSITE consensus patterns (Bairoch, 1992) are shown by the shaded residues with their names under the sequences.
**A.** At3g12500 or ATHCHIB (Samac *et al*., 1990). "Chitin-binding" stands for Chitin recognition or binding domain signature PS00026 (C-x(4,5)-C-C-S-x(2)-G-x-c-g-x(4)-[FYW]-C); (1) for Chitinase 19_1 signature PS00773 (C-x(4,5)-F-Y-[ST]-x(3)-[FY]-[LIVMF]-x-A-x(3)-[YF]-x(2)-F-[GSA]) and (2) for Chitinase 19_2 signature PS00774 ([LIVM]-[GSA]-F-x-[STAG](2)-[LIVMFY]-W-[FY]-W-[LIVM]). "CTE" stands for C-terminal extension. The residues in bold are essential for catalytic activity, the residues marked with an asterisk are important for catalytic activity, the boxed residues putatively bind the substrate and the active sites are indicated by the bars under the sequence (Garcia-Casado *et al*., 1998). The tyrosine residue indicated by the arrow is essential for substrate binding in the catalytic site but not for catalysis (Verburg *et al*., 1993; Verburg *et al*., 1992). **B.** At5g20490 or ATHCHIA (Samac *et al*., 1990). (18) stands for Chitinase_18 signature PS01095 ([LIVMFY]-[DN]-G-[LIVMF]-[DN]-[LIVMF]-[DN]-x-E). As in (A), residues in bold are essential for catalytic activity (Watanabe *et al*., 1993).

ed to the vacuole by means of the C-terminal extension (Neuhaus *et al*., 1991b and Figure 4A), although there is no immunocytological evidence for the latter. Based on the nature and presence of an N-terminal signal sequence the protein could also be apoplastic (Figure 4A and Table 2). Its expression was shown to be regulated in an age-dependent and tissue-specific manner. Predominantly expressed in roots of untreated plants, the gene is also expressed in leaves and flowers of aging plants and is not induced upon wounding, excluding a role in a general stress-response (Samac *et al*., 1990). Furthermore, its expression can be enhanced by ethylene, which probably also corresponds to increasing ethylene levels in aging plants and a possible link with senescence in leaves and flowers. It was proposed that the constitutive expression in roots is not controlled by ethylene, since the gene remains expressed in roots of ethylene insensitive mutants (Samac *et al*., 1990). It could be that the ATHCHIB chitinase has multiple functions at different stages of plant development, some of which might be regulated by ethylene. This was indeed demonstrated in several studies linking induction of this chitinase and ethylene-controlled processes such as seedling growth (Chen and Bleecker, 1995; Larsen and Chang, 2001). In addition, the role that the basic chitinase could play in plant defense also seems to be controlled by ethylene. Purified ATHCHIB chitinase could inhibit the growth in vitro of the fungus *Trichoderma reesei*, but not of any of the other fungi tested, suggesting a rather specific pathogen-dependent defense response (Verburg and Huynh, 1991). However, Thomma *et al*. (1999) also clearly showed that ethylene is required for the induction of the ATHCHIB chitinase upon fungal infection and consequently for resistance against the fungus. This study also confirmed the pathogen-specificity of this response. Therefore, the *Arabidopsis* class I chitinase is likely to be activated by an ethylene-dependent signaling pathway and may function in plant defense against specific strains of fungi, perhaps based on its primary role in controlling senescence.

### *5.2. Class II*

Class II chitinases are represented by four members in *Arabidopsis*, none of which has been studied so far. Two sequences (At1g05870 and At3g16920) are not likely to be active as chitinases, since they are missing some of the amino acid residues essential for catalytic activity (Figure 5). Yet they are actively transcribed and could therefore have an alternative function, which cannot presently be deduced from their sequences. It is also not possible to derive any function from the sequences to which they are the most similar (Table 2), i.e. a potato class II chitinase (Wemmer *et al*., 1994) and a tomato class II chitinase (Danhash *et al*., 1993) since these possess all essential residues. It is therefore likely that the two *Arabidopsis* genes have another unknown function. The two other *Arabidopsis* class II chitinases (At1g02360 and At4g01700) on the other hand have all necessary residues to act as chitinases (Figure 5) that are most likely secreted (Table 2). Based on the homology they share with chitinases from other plants we can hypothesize what their function could be (Table 2). For example class II chitinase Ch2;1 from peanut is exclusively expressed upon treatment with fungal spores whereas the gene encoding the isoform Ch2;2 appears to be constitutively expressed but is inducible by treatment with ethylene, salicylic acid or fungal spores (Kellmann *et al*., 1996). In parsley, a similar situation is found with differential expression of two class II isoforms (Kirsch *et al*., 1993; Ponath *et al*., 2000). The gene encoding one of the isoforms is highly induced whereas the gene encoding the other one is only moderately induced upon fungal infection. Both genes are also constitutively expressed in different organs of healthy plants, and it was proposed that they could play distinct roles during plant defense but also have distinct endogenous regulatory functions in plant development (Ponath *et al*., 2000). Similarly to class I chitinases, class II chitinases may have multiple functions depending, on the isoform but also depending on the stage of development. Based on the data of the peanut and parsley chitinases, we can also propose that one *Arabidopsis* isoform is probably specialized in defense against a few specific pathogens as well as in development, whereas the other isoform is probably involved in a more general stress response. The absence of a chitin-binding domain in class II chitinases also suggests that they are most likely acting on different substrates and/or in different contexts than class I chitinases.

### *5.3. Class III*

The only class III chitinase in *Arabidopsis*, ATHCHIA (At5g24090) was also isolated and studied by Samac *et al*. (1990). It is a secreted acidic chitinase (Table 2), of which the gene also appears to be developmentally regulated as well as induced by pathogens (Samac and Shah, 1991). Based on promoter::b-glucuronidase (GUS) studies, the class III chitinase is expressed in roots, leaf vascular tis-

sue, hydathodes, guard cells and anthers of healthy plants and is also induced in mesophyll cells surrounding lesions caused by fungal infection (Samac and Shah, 1991). The same study showed that the induction was dependent on the fungal strain used and that it was neither ethylene- nor salicylic acid- or wounding-dependent. This suggests a rather specific activation that is probably synonymous with a direct action at the infection site, as also suggested by the expression in cells directly around necrotic lesions (Samac and Shah, 1991). In contrast with the class I chitinase ATHCHIB, ethylene signaling does not seem to be involved here, and activation must rely on a different signaling molecule, such as an elicitor from specific fungi. The exact mode of action of the acidic chitinase is unknown, and the use of antisense suppression did not provide more clues on the matter. Plants with chitinase levels reduced to less than 10% that of the wild-type showed no sign of increased susceptibility to fungal infection (Samac and Shah, 1994). This suggests that since ATHCHIA is a single copy gene (Samac *et al*., 1990) and encodes the only *Arabidopsis* class III chitinase, chitinases from other classes are probably able to take over its function. Furthermore, no morphological phenotype was described for the antisense plants (Samac and Shah, 1994). So this probably holds for pathogen-response as well as development and lends support to the apparent multifunctionality of plant chitinases that seem to be functionally interchangeable from one class to another.

### *5.4. Class IV*

The members of class IV represent, together with class V, the majority of the *Arabidopsis* chitinases. Among the nine sequences that show all structural characteristics of class IV chitinases, four encode apparently inactive chitinases lacking essential amino acid residues (Figure 6). All four are not likely to be transcribed and probably correspond to pseudogenes. The other five sequences are most likely secreted active chitinases. So far, only one of them, At3g54420 encoding AtEP3/AtchitIV, is being studied (de A. Gerhardt *et al*., 1997; Passarinho *et al*., 2001) and as found for the other classes, all experiments suggest multiple functions. The detailed analysis of the AtEP3/AtchitIV expression pattern using promoter::GUS fusions revealed that the gene is spatially and temporally regulated. In tissue-culture, it is specifically expressed in embryogenic cultures. In planta it is expressed in mature and germinating pollen, in growing pollen tubes, in the seed coat or the endosperm cap during germination, in growing root hairs and in leaf hydathodes and stipules (Passarinho *et al*., 2001). This is strikingly similar to what was found for the class III chitinase gene (Samac and Shah, 1991). Based on previous work done in carrot (de Jong *et al*., 1992; van Hengel *et al*., 1998; van Hengel *et al*., 2001), it is very likely that the AtEP3/AtchitIV chitinase is involved in embryo development, and may also act via GlcNAc-containing signal molecules (de Jong *et al*., 1993). Such signaling molecules could be released by cleavage of specific types of arabinogalactan proteins (AGPs; van Hengel *et al*., 2001), which suggested that there are indeed plant substrates for endochitinase activity. AGPs and chitinases have been co-localized in several plant tissues. AGPs are found in the style of several plant species (Cheung *et al*., 1995; Du *et al*., 1996; Lind *et al*., 1994), just as chitinases (Leung, 1992; Takakura *et al*., 2000; Wemmer *et al*., 1994), and stylar AGPs were shown to play a role in pollen-stigma interactions as well as during pollen tube growth (Cheung *et al*., 1995). Chitinases present in pollen and/or in the stigma could therefore contribute to the same processes by AGP processing.

The analysis of total AGP content, crossed electrophoresis patterns, RNA blots, and western blots showed that AGP expression is both quantitatively and qualitatively regulated during germination and seedling development (Lu *et al*., 2001). AGPs are also present in the root epidermis (Samaj *et al*., 1999) and are involved in root and root hair development (Ding and Zhu, 1997; Willats and Knox, 1996). These observations may indicate that AGP processing by chitinases is a widespread phenomenon.

A role for class IV chitinases in plant defense was also proposed by de A. Gerhardt *et al*. (1997). But most evidence comes from work done on other plant species where it was clearly shown that the expression of some class IV chitinases was induced upon fungal infection and could be associated with plant resistance (Lange *et al*., 1996; Nielsen *et al*., 1994; Rasmussen *et al*., 1992). Class IV chitinases also respond to a broader range of stress sources, like virus infection, heavy metals and UV irradiation (Margis-Pinheiro *et al*., 1993). This suggests that the specificity towards pathogens found with the ATHCHIB class I chitinase (Verburg and Huynh, 1991) and the ATHCHIA class III chitinase (Samac and Shah, 1991) may be less restricted in class IV chitinases. In other plant species, a role in senescence was suggested based on the high levels of class IV chitinase expression found in senescing *Brassica* leaves (Hanfrey *et al*., 1996), ripening grape berries (Robinson *et al*., 1997) or banana fruits (Clendennen and May, 1997). This may point to a link between class IV chitinases and induction by ethylene. Ethylene is often associated with fruit maturation and aging (Payton *et al*., 1996) but also with programmed cell death (Greenberg and Ausubel, 1993). In conclusion, it is clear that class IV chitinases may also have multiple func-

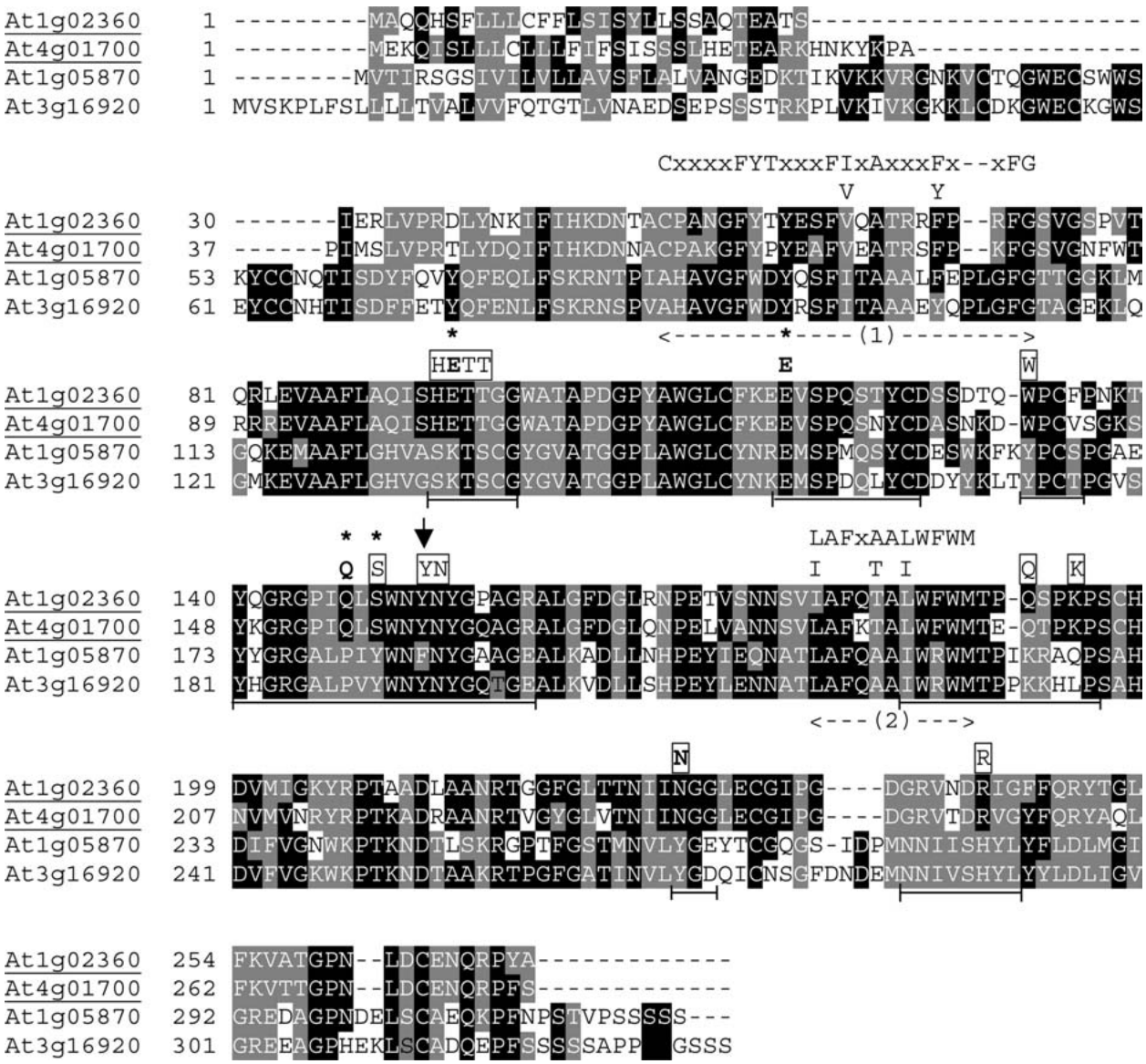



**Figure 5. Multiple sequence alignement of Arabidopsis class II chitinases.**
Gaps were introduced for optimal alignment and the degree of shading represents the level of similarity. PROSITE consensus patterns (Bairoch, 1992) are indicated above the aligned sequences and their names under. (1) stands for Chitinase 19_1 signature PS00773 (C-x(4,5)-F-Y-[ST]-x(3)-[FY]-[LIVMF]-x-A-x(3)-[YF]-x(2)-F-[GSA]) and (2) for Chitinase 19_2 signature PS00774 ([LIVM]-[GSA]-F-x-[STAG](2)-[LIVMFY]-W-[FY]-W-[LIVM]). In class I chitinases, the residues in bold are essential for catalytic activity, the residues marked with an asterisk are important for catalytic activity, the boxed residues putatively bind the substrate and the active sites are indicated by the bars under the sequence (Garcia-Casado *et al*., 1998). The tyrosine residue indicated by the arrow is essential for substrate binding in the catalytic site but not for catalysis (Verburg *et al*., 1993; Verburg *et al*., 1992). The underlined accessions possess all required characteristics for chitinase activity.

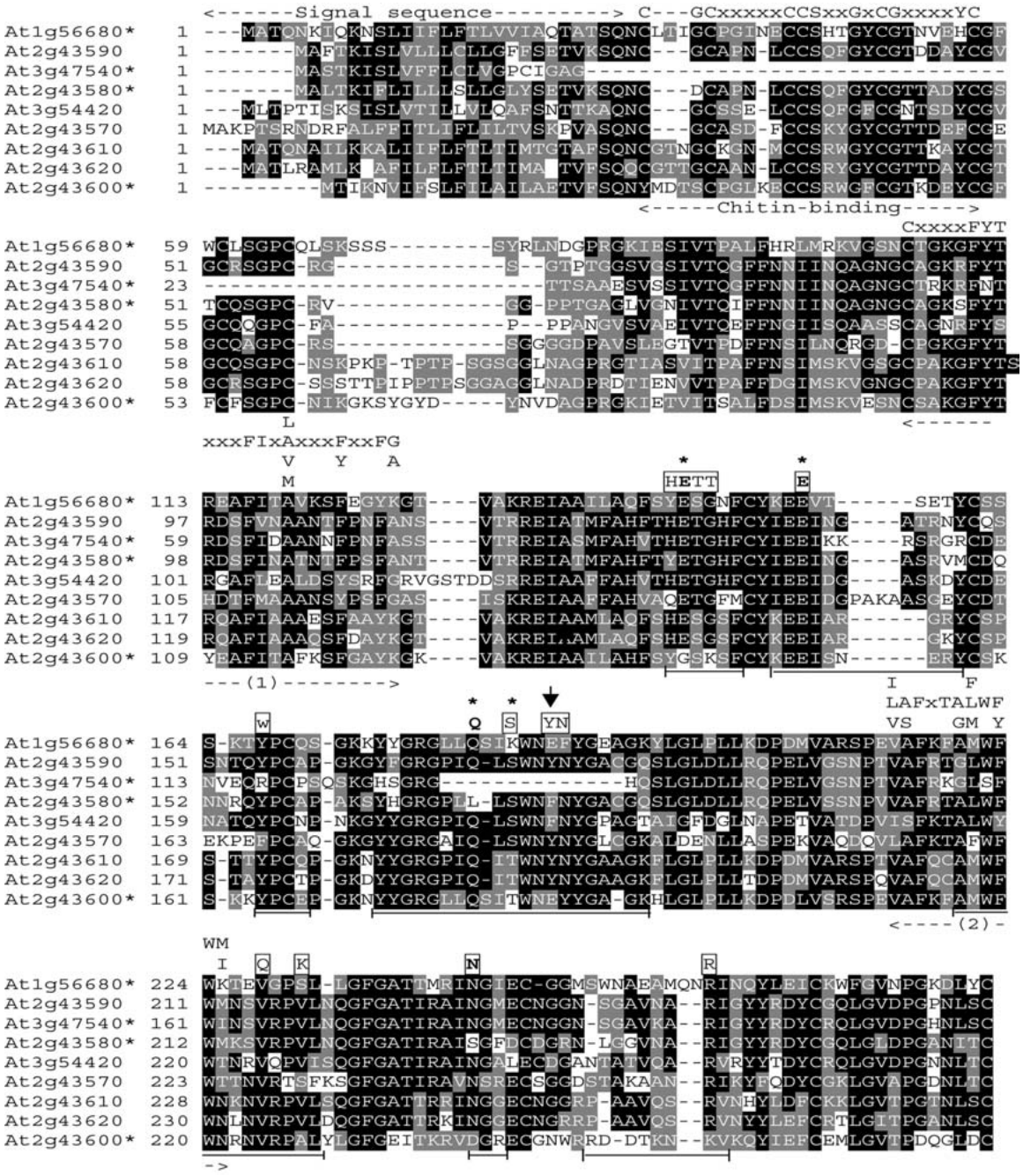


**Figure 6. Multiple sequence alignment of Arabidopsis class IV chitinases.**
Gaps were introduced for optimal alignment and the degree of shading represents the level of similarity. The (*) marks the putative genes, for which no EST were found. PROSITE consensus patterns (Bairoch, 1992) are indicated above the aligned sequences and their names under. "Chitin-binding" stands for Chitin recognition or binding domain signature PS00026 (C-x(4,5)-C-C-S-x(2)-G-x-c-g-x(4)-[FYW]-C); (1) for Chitinase 19_1 signature PS00773 (C-x(4,5)-F-Y-[ST]-x(3)-[FY]-[LIVMF]-x-A-x(3)-[YF]-x(2)-F-[GSA]) and (2) for Chitinase 19_2 signature PS00774 ([LIVM]-[GSA]-F-x-[STAG](2)-[LIVMFY]-W-[FY]-W-[LIVM]). In class I chitinases, the residues in bold are essential for catalytic activity, the residues marked with an asterisk are important for catalytic activity, the boxed residues putatively bind the substrate and the active sites are indicated by the bars under the sequence (Garcia-Casado *et al*., 1998). The tyrosine residue indicated by the arrow is essential for substrate binding in the catalytic site but not for catalysis (Verburg *et al*., 1993; Verburg *et al*., 1992).

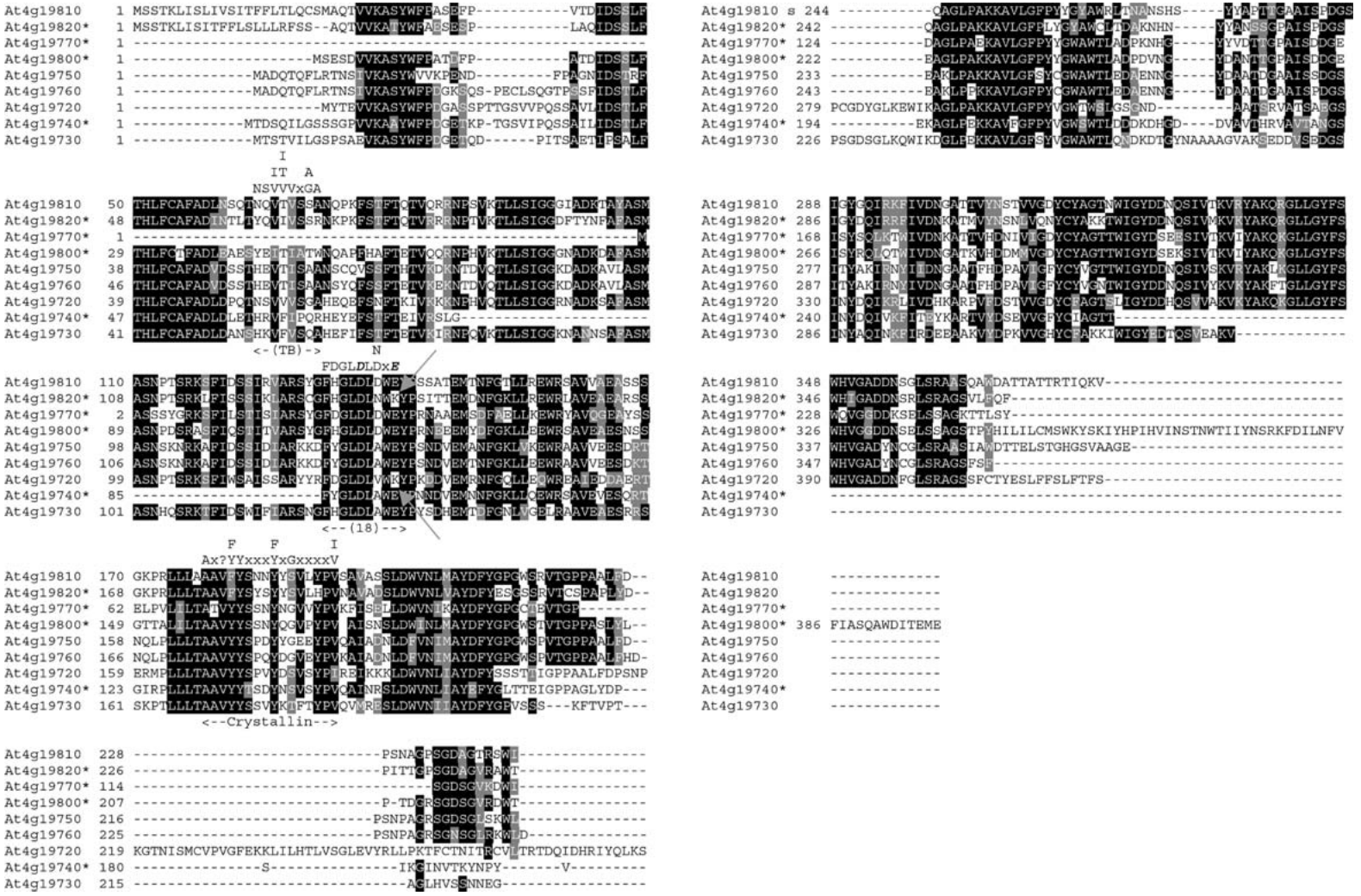



**Figure 7. Multiple sequence alignement of Arabidopsis class V chitinases.**

Gaps were introduced for optimal alignment and the degree of shading represents the level of similarity. The (*) marks the putative genes, for which no ESTs were found. PROSITE consensus patterns (Bairoch, 1992) are indicated above the aligned sequences and their names under. (TB) stands for TONB_DEPENDENT_REC1 signature PS00430 (x(10,115)-[DENF]-[ST]-[LIVMF]-[LIVSTEQ]-V-x-[AGP]-[STANEQPK]); (18) stands for Chitinase_18 signature PS01095 ([LIVMFY]-[DN]-G-[LIVMF]-[DN]-[LIVMF]-[DN]-x-E) and (Crystallin) for CRYSTALLYN_ BETAGAMMA signature PS00225 ([LIVMFYWA]-{DEHRKSTP}-[FY]- [DEQHKY]-x(3)-[FY]-x-G-x(4)-[LIVMFC-ST]). The residues in bold and italic above the alignment are essential for catalytic activity (Watanabe *et al*., 1993). The gray arrows indicate a lysine residue differing from the expected essential glutamic acid, which resembles what is found in concanavalin B (Hennig *et al*., 1995).

tions, but in *Arabidopsis* it seems that these proteins may be more involved in developmental processes rather than in defense reactions.

### *5.5. Class V*

As in class IV, nine sequences were found in the *Arabidopsis* genome that showed the structural features of class V chitinases (Figure 7). Among those, two (At4g19720 and At4g19820) appear to be non-active chitinases from family 18 of glycosyl hydrolases since they lack the essential glutamic acid of the catalytic site (Figure 7). This resembles concanavalin B (Hennig *et al*., 1995), a gene that is actively transcribed and produces a protein that is a close relative of family 18 chitinases but does not possess any chitinase activity. Concanavalin B may have a function in the storage of seed carbohydrates. This is interesting, especially since one of the *Arabidopsis* class V transcribed sequences, At4g19720, contains a motif specific for narbonin (Table 2) another concanavalin B-like molecule (Nong *et al*., 1995). At4g19720 also has a motif specific for TonB (Figure 7 and Table 2). TonB is a bacterial receptor-associated protein, that is involved in active transport of poorly permeable substrates through the membrane (Gudmundsdottir *et al*., 1989). This could indicate that this chitinase-like protein might be involved in the perception and recruiting of specific chitin-derived molecules in order to allow their transport into the cell for subsequent processing by active chitinases. Or they could participate in the perception of these molecules by a specific-receptor and thereby activate a signaling cascade leading to a morphological process or a defense response. This is particularly interesting in the light of the work recently published by Day *et al.* (2001), showing that specific chitin-binding sites are present in the plasma membrane of soybean. A previous study in rice had also shown the presence in the plasma membrane of suspension-cultured cells of a high-affinity binding protein for a N-acetylchitooligosaccharide elicitor (Ito *et al*., 1997). This could be in agreement with the identification in tobacco of a receptor kinase with an extracellular domain similar to a class V chitinase that, as concanavalin B (Hennig *et al*., 1995), lacks the essential glutamic acid of the catalytic site (Kim *et al*., 2000). It is noteworthy that At4g19820, the second *Arabidopsis* concanavalin B-like protein, although it has a sequence highly similar to At4g19720, does not possess a narbonin or a TonB motif (Figure 7 and Table 2). Moreover At4g19820 is not likely to be transcribed, which suggests that in At4g19720, the narbonin or TonB motifs may be functionally relevant, implying a receptor-like function. All other class V sequences possess all the essential amino acid residues for catalytic activity and are therefore probably active chitinases (Figure 7). However, they are most likely involved in different mechanisms since they are targeted to different cell compartments (Table 2). For example, At4g19750 and At4g19760 that are actively transcribed class V chitinase sequences contain a nuclear localization signal. They also contain an additional motif specific for crystallins (Table 2). Crystallins are the main constituent of the eye lens but the corresponding motif is also found in dormancy proteins of some microorganisms (Wistow, 1990). Dormancy proteins are activated in response to various kinds of stress. The relation between the crystallin motif and a nuclear localization is unclear, but could point to a role in modifying the cell cycle or in inducing programmed cell death. Two other members (At4g19770 and At4g19800) contain a similar crystallin-like motif, but none of these two class V chitinase sequences is likely to be transcribed, furthermore they lack a nuclear localization signal (Table 2). The other members of class V are either secreted (At4g19810) or targeted to the peroxisomes (At4g19730 and At4g19740). In conclusion, class V chitinases represent a rather diverse group of chitinases and very little is known about their functional aspects. In tobacco it was shown that they may be involved in plant defense but that they are also developmentally regulated (Heitz *et al.*, 1994; Melchers *et al*., 1994). The class V chitinases that resemble concanavalin B could be involved in chitin perception and recruiting following the model proposed for the CHRK1 receptor from tobacco (Kim *et al*., 2000).

## 6. Conclusions.

Sequencing and systematic automated annotation of the *Arabidopsis* genome has led to the classification of 24 sequences as putative chitinase-encoding genes. A more detailed analysis of the individual sequences reveals one of the limitations of large-scale automated genome annotation. Sequence details that are functionally important can be missed because at present it is difficult to incorporate an integrated view of all data available on protein families into the annotation software. Indeed, out of the 24 chitinase sequences, 8 are not likely to be transcribed while 3 others do not contain amino acid residues that are essential for catalytic activity. Consequently, they probably have a function different from the hydrolysis of chitin-derived molecules. This is also true for most of the sequences for which no ESTs were found.

The genomic distribution of the chitinase-encoding

genes shows a remarkable degree of clustering per class (class IV on chromosome II and class V on chromosome IV; Figure 8). Similar genes are indeed repeated in tandem but also duplicated on other chromosomal regions like At1g02360 and At3g16920. This reflects one of the characteristics of the *Arabidopsis* genome, that is largely made up of duplicated chromosomal regions (Blanc *et al*., 2000; Vision *et al*., 2000). Chitinase genes belong to relatively large families (Graham and Sticklen, 1994) that are probably the result of such duplication events.

Chitinases are grouped into five different classes that differ in sequence, 3D structure and biochemical properties (Neuhaus *et al.*, 1996). In *Arabidopsis*, as in all other plants studied so far, chitinases of each class are present. These are rather equally represented, if one removes all sequences that are most likely not transcribed (Figure 8), and it is reasonable to assume that they have developed class-specific functions, especially between chitinases of family 18 and 19. Furthermore, the analysis we performed here reveals that there are also differences between related classes such as class I and class IV as well as within classes, like in classes II and V. This is probably indicative of different substrate specificities and thereby suggest a rather high degree of specialization. It is also clear that most chitinases, independently from their class, are probably involved in several functions.

Some chitinases (e.g. *Arabidopsis* classes I and III (Samac *et al.*, 1991; Verburg and Huynh, 1991) and some isoforms of class II, e.g. in parsley (Ponath *et al.*, 2000) and peanut (Kellmann *et al*., 1996)) are only activated upon

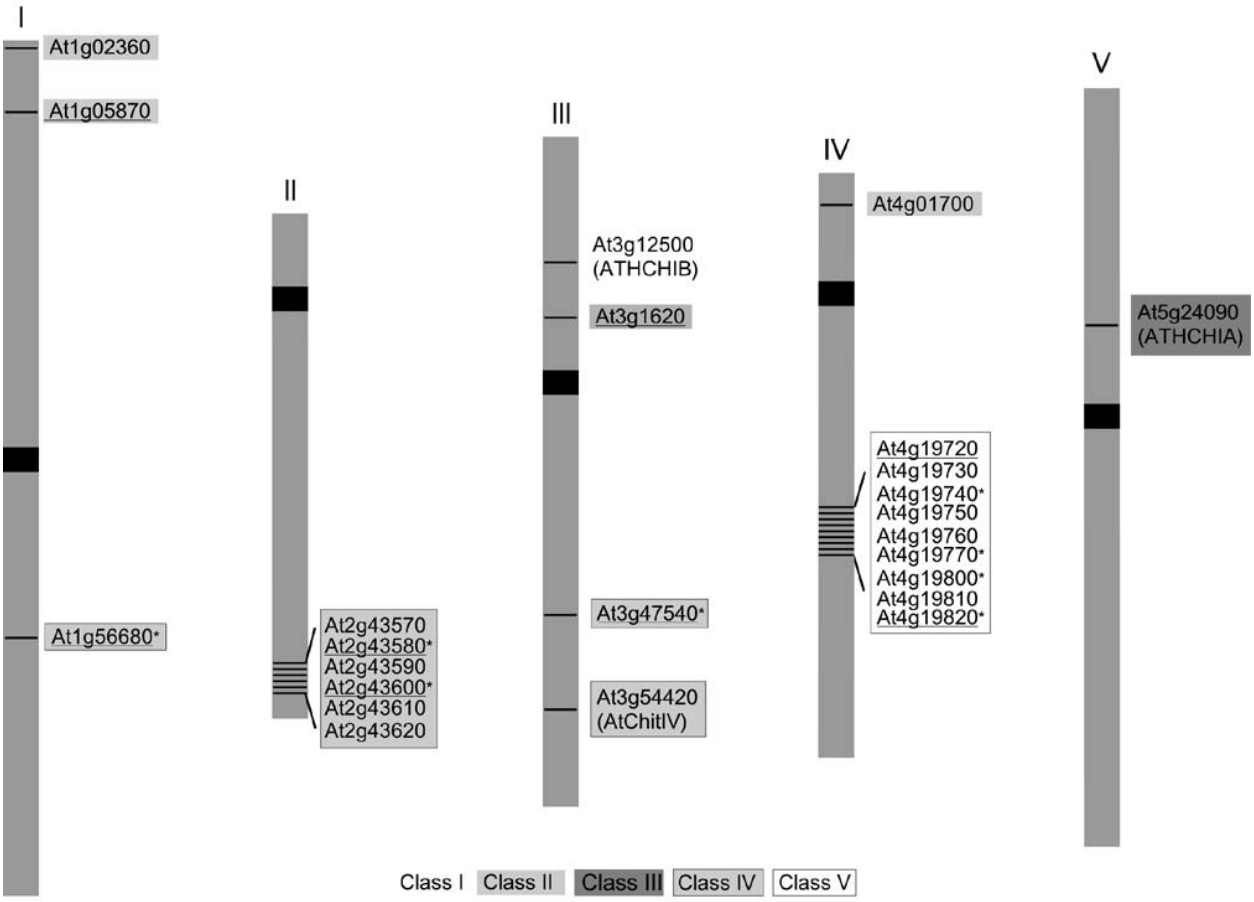



**Figure 8. Recapitulation of the characteristics of the Arabidopsis chitinase annotations.**
As in Figure 1, the locus of each annotation is indicated on the five *Arabidopsis* chromosomes. The (*) indicates sequences that are not likely to be transcribed. The degree of shading and the boxes around the locus names represent the belonging class of the corresponding sequence and those that are underlined miss some of the amino acid residues essential for chitinase activity

infection with specific strains of fungi, implying a role in a highly specialized defense response. Others (e.g. bean class IV (Margis-Pinheiro *et al*., 1993) and some isoforms of class II, e.g. in parsley (Ponath *et al.*, 2000) and peanut (Kellmann *et al*., 1996)) seem to be involved in more general stress responses that do not require a very specific interaction with a pathogen. Furthermore, their range of action in response to pathogen infection also seems to be different. Classes III and V chitinases that belong to the glycosyl hydrolase family 18, seem to be involved in a short-range response that suggests a direct action on the invading pathogen.

The *Arabidopsis* class III chitinase ATHCHIA that is induced by very specific strains of pathogens and does not seem to require any other form of signaling (e.g. ethylene) for activation, is a typical example. This is supported by its activation directly at the infection site (Samac and Shah, 1991). Furthermore, the inactive chitinases of the concanavalin B-type found in class V suggest a putative role in the perception and recruitment of chitin-derived molecules (Hennig *et al*., 1995; Kim *et al.*, 2000). This may strengthen the idea of a direct interaction with the invading pathogen. And last, the additional lyzosymal activity that is characteristic of these two classes combined with the putative localization of some isoforms in the peroxisomes could also indicate an activity involved in direct degradation of the pathogen. Genes of the other classes are more likely to be activated indirectly via a signaling cascade triggered upon identification of a specific pathogen by, for example, a class V chitinase of the concanavalin B-type. This is probably the case for the *Arabidopsis* class I chitinase ATHCHIB and for some specific isoforms of class II (Kellmann *et al*., 1996; Ponath *et al.*, 2000). Other isoforms of class II as well as class IV chitinases are probably activated by more general forms of stress that eventually may lead to the same general response. Plant hormones, such as ethylene, may be the mediators of these signaling events.

The role ethylene plays in development also brings us to the developmental regulation of chitinase genes. This seems to be valid for all classes and their exact function at this level is probably determined by the part of the plant in which they are localized and on the available substrates. These substrates can be of a symbiotic origin (rhizobial Nod factors) that upon perception and processing by chitinases are able to trigger a cascade of specific events leading to the formation of a root nodule (Ovtsyna *et al*., 2000). Alternatively, substrates must be of plant origin, implying the existence of plant endogenous GlcNAc-containing molecules. Recent work has demonstrated that these molecules could be AGPs (van Hengel *et al.*, 2001). This is in line with the large distribution of AGPs in different plant tissues (Knox, 1999) and their great plasticity in carbohydrate composition. Thus, GlcNAc- or GlcN-containing AGPs could exist in many plant organs and provide highly specific substrates to matching specific chitinases.

In conclusion, it is clear that the function of plant chitinases is still poorly understood. Chitinases seem to be involved in many different aspects of the plant life cycle, and it will be difficult to dissect such aspects in great detail. Understanding the role of plant chitinases will require the generation of mutant plants that lack one or several specific chitinases, to create a background with different combinations of chitinases and circumvent problems of gene redundancy but also to understand the specific interrelations between the different classes. It will also imply the combined study of the role of AGPs following similar approaches and most certainly detailed immunocytological and biochemical studies to unravel the complex chitinase-AGP combinations in association with very specific processes.

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