Functional Analysis of the *Arabidopsis thaliana* AtEP3 Endochitinase

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Statements

1. Accurate automated large-scale genome annotation remains an illusion.

2. "Gene knockout alone is not sufficient to assess gene function." Bouché, N. and Bouchez, D. (2001) *Curr. Opin. Plant Biol.*, 4: 111-117.

3. Plant chitinases are multifunctional proteins that are not only involved in plant defense. This thesis.

4. "Experience is something that you only acquire after needing it." Murphy's Law, Arthur Bloch, 1990.

5. "An expert is someone that has made all possible mistakes in a given field." Murphy's Law, Arthur Bloch, 1990.

6. "The Dutch language in its written form looks like someone sat on a typewriter." The Dutch Courier, Australia.

7. "The more you try to learn Dutch the more the Dutch refuse to speak Dutch to you." The Undutchables, 3rd Edition, 1993.

8. "Having a smoking section in a restaurant is like having a peeing section in a swimming-pool." Anonymous.

Statements from the thesis entitled:
"Functional Analysis of the *Arabidopsis thaliana* AtEP3 Endochitinase"
Paul Passarinho, Wageningen, 12 December 2001.

Contents

Outline

Plant chitinases (EC 3.2.1.14) are abundant proteins found in all plants studied to date. They belong to the large family of glycosyl hydrolases and are able to catalyze the hydrolysis of chitin that is a major component of the insect exoskeleton and of the cell wall of many fungi. The results of numerous studies have suggested that chitinases are involved in plant defense against fungal pathogens but also in more general stress responses induced for example upon wounding, treatment by salicylic acid, plant hormones, heavy metals or UV irradiation. Furthermore, it has become apparent that most chitinases are also developmentally regulated; specific isoforms are being differentially expressed in particular plant organs at defined developmental stages. However, it still remains unclear whether such specific expression is related to a role in development or whether it reflects a preparation to a potential defense response in these organs. Yet there is evidence of an active role of chitinases in development. Tobacco plants overexpressing a maize chitinase gene were shown to grow taller and thicker than wild-type plants. But the most direct evidence comes from work done in carrot that revealed that the EP3 endochitinase was able to rescue the arrested embryos of the temperature sensitive mutant *ts11*. Further work has demonstrated that this action was probably mediated by the processing of signal molecules that contain N-acetylglucosamine (GlcNAc), the basis of chitin, since specific rhizobial Nod factors displayed the same rescue activity on *ts11* embryos. This implied the existence in plants of a similar endogenous substrate that could be used by chitinases. Recent work has shown that a possible substrate could be specific arabinogalactan proteins (AGPs), since they can be processed by the EP3 chitinase. Since more than one chitinase was shown to be able to lift the embryo development arrest imposed on the *ts11* mutant, the *ts11* phenotype appears to be indirectly linked to the EP3 chitinase. In addition, it appeared that the EP3 chitinase was the member of a family with at least five different isoforms. It was therefore becoming clear that studying the role of the EP3 chitinase in carrot embryo development using a genetic approach would not be an easy task.

To avoid some of the expected difficulties it was decided to pursue a reverse genetic approach in *Arabidopsis thaliana*. The small size of its genome combined with all the genetic tools developed by the *Arabidopsis* community were encouraging elements to search for "the" single copy gene that was homologous to the most active carrot EP3 chitinase isoform. The search for and the subsequent study of the *Arabidopsis* EP3 gene are described in this thesis.

In Chapter 1, an overview of all *Arabidopsis* chitinase genes is presented. This chapter follows the release of the complete sequence of the *Arabidopsis* genome and discusses the features of all sequences annotated as chitinase. Modified annotations are proposed and possible functions are discussed based on the characteristics of each sequence and in relation with other plant chitinases.

In Chapter 2, the cloning and the characterization of the *Arabidopsis* ortholog of the carrot EP3 chitinase gene are described. The physical and the genetic mapping of the gene locus are presented as well, together with a detailed analysis of it expression pattern in planta and in vitro, based on RT-PCR and promoter::reporter fusions.

In Chapter 3, the results of a study on the expression of specific gametophytic markers during *Arabidopsis* somatic embryogenesis are presented. This work was carried out in relation to the specific expression of the *AtEP3* gene during pollen development in planta and during somatic embryogenesis. A number of highly specific GUS markers for the male and the female gametophyte were therefore studied during somatic embryogenesis to verify if gametophytic gene expression programs are conserved during somatic embryogenesis.

In Chapter 4, the first functional analysis of a chitinase gene by insertional mutagenesis is presented. The details of the screening procedure as well as the subsequent analyses are described. The nature of the material and the conditions in which it was studied did not allow to determine the function of the AtEP3 chitinase.

In Chapter 5, the results of a transgenic approach involving the overexpression and the antisense suppression of the *AtEP3* gene are described. The production and the analysis of transgenic plants with altered expression levels of *AtEP3* mRNA are presented and reveal the involvement of the AtEP3 chitinase in seed and root hair development. An additional male sterile mutant was also identified and is described.

In Chapter 6, the results of a successful reverse genetics approach are presented. An insertion mutant was identified for the *AtEP3* gene and the preliminary results of its molecular and phenotypic characterization are described. These results lend support to previous

hypotheses and confirm a role of the chitinase in seed germination, pollen and root hair development.

In Chapter 7, the results of this research are compiled in a summarizing discussion emphasizing on the role of the AtEP3 chitinase during development in relation with putative substrate molecules.

Chapter 1

Arabidopsis chitinases: a genomic survey

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.

Paul A. Passarinho and Sacco C. de Vries

1. Introduction

Chitinases (EC 3.2.1.14) are classified as glycosyl hydrolases and catalyze the degradation of chitin, an insoluble linear β-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. β-anomeric form) whereas family 19 members use an inversion mechanism turning the product into the α-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, Absidiomycetes 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 β-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 studied in this thesis 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).

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 gi38559599	Putative chitinase	280	31.5	10	Biogenesis of cell wall (MATDB)	II

Table 1. *Arabidopsis* chitinase annotations (continued).
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 locus name in bold indicates the AtEP3 chitinase studied in this thesis. 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 "PZ-precursor" (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.

Locus (Clone name)	Chr	Accessions	Annotation	Length (aa)	MW (kDa)	ESTs	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

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

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).

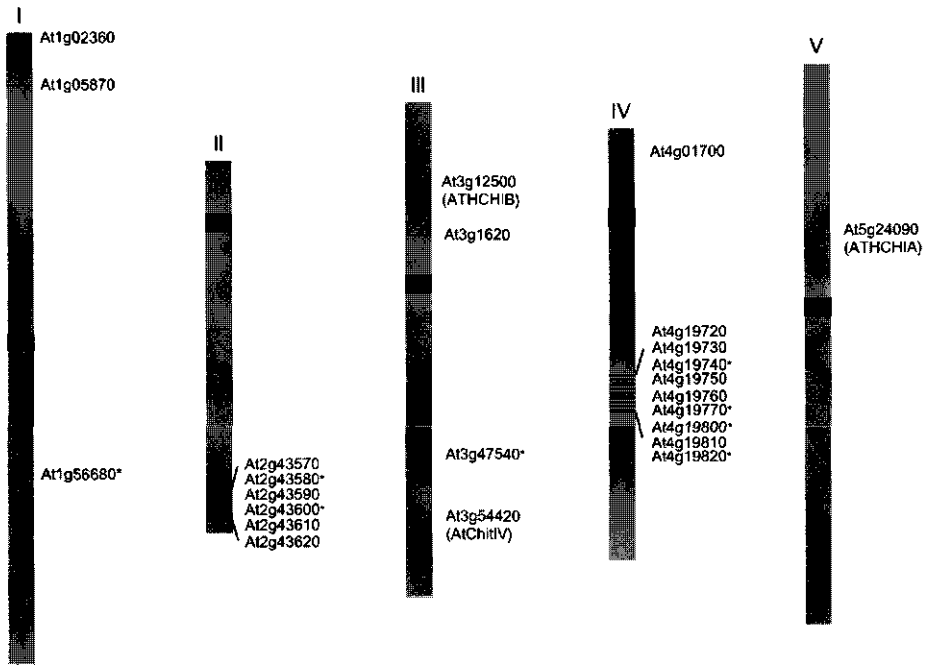


Figure 1. Genomic distribution of the *Arabidopsis* chitinase-encoding genes.
The locus of each accession is shown on the individual chromosomes. The (*) marks the putative genes, for which no ESTs were found.

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).

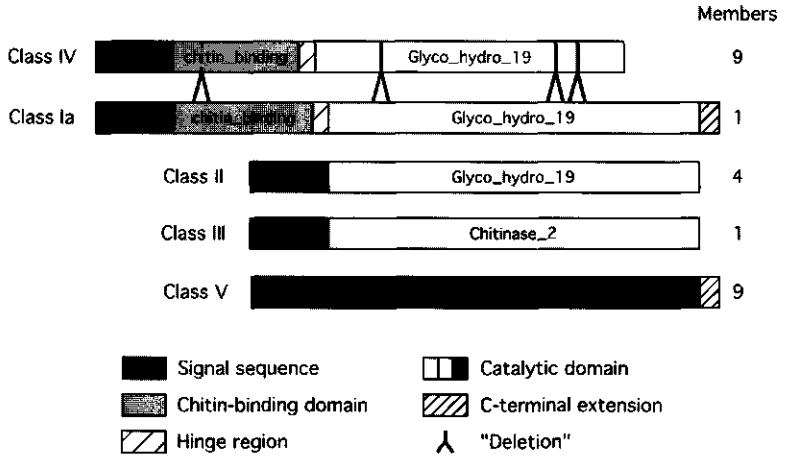


Figure 2. Classification and structure of the chitinase proteins found in the *Arabidopsis* genome.
The structural domains are schematically represented and include the names of the corresponding signatures found in the Pfam protein families database (Bateman *et al.*, 2000). Chitin_binding corresponds 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).

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).

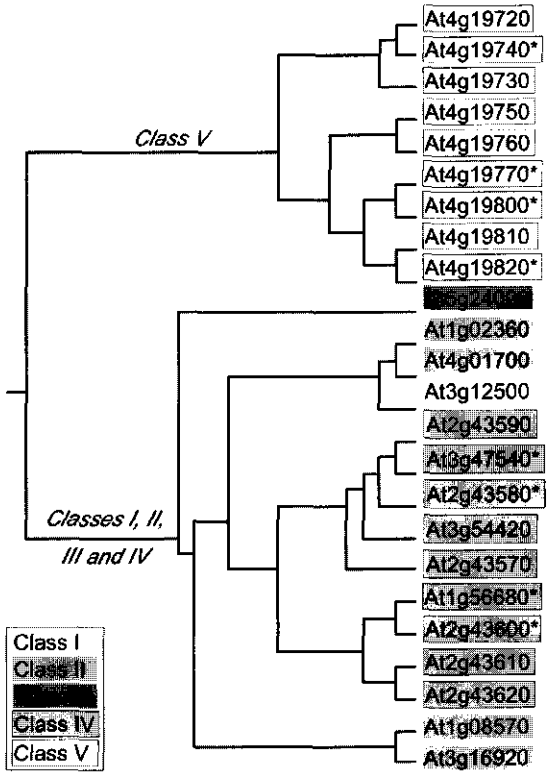


Figure 3. Phylogenetic tree of the *Arabidopsis* chitinase proteins.
The dendrogram was generated by using the CLUSTALW Multiple Sequence Alignment program at the GenomeNet WWW server (http://clustalw.genome.ad.jp/). The belonging classes of each accession are indicated by the shading and boxes around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

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.

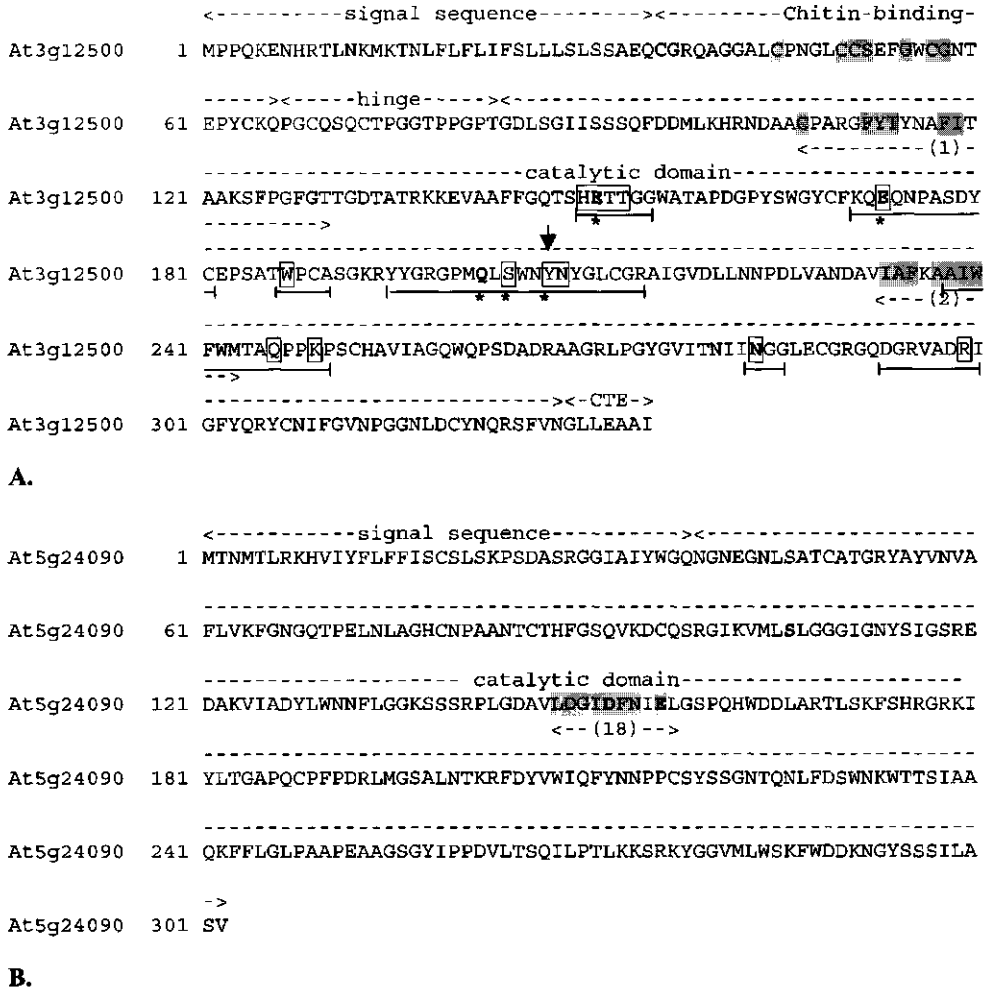
Figure 3 shows the phylogenetic tree generated with the 24 sequences by using the CLUSTALW Multiple Sequence Alignment program at the GenomeNet WWW server (http://clustalw.genome.ad.jp/). The different classes are nicely clustered and it is clear that class V has diverged from the other classes very 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 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



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).

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).

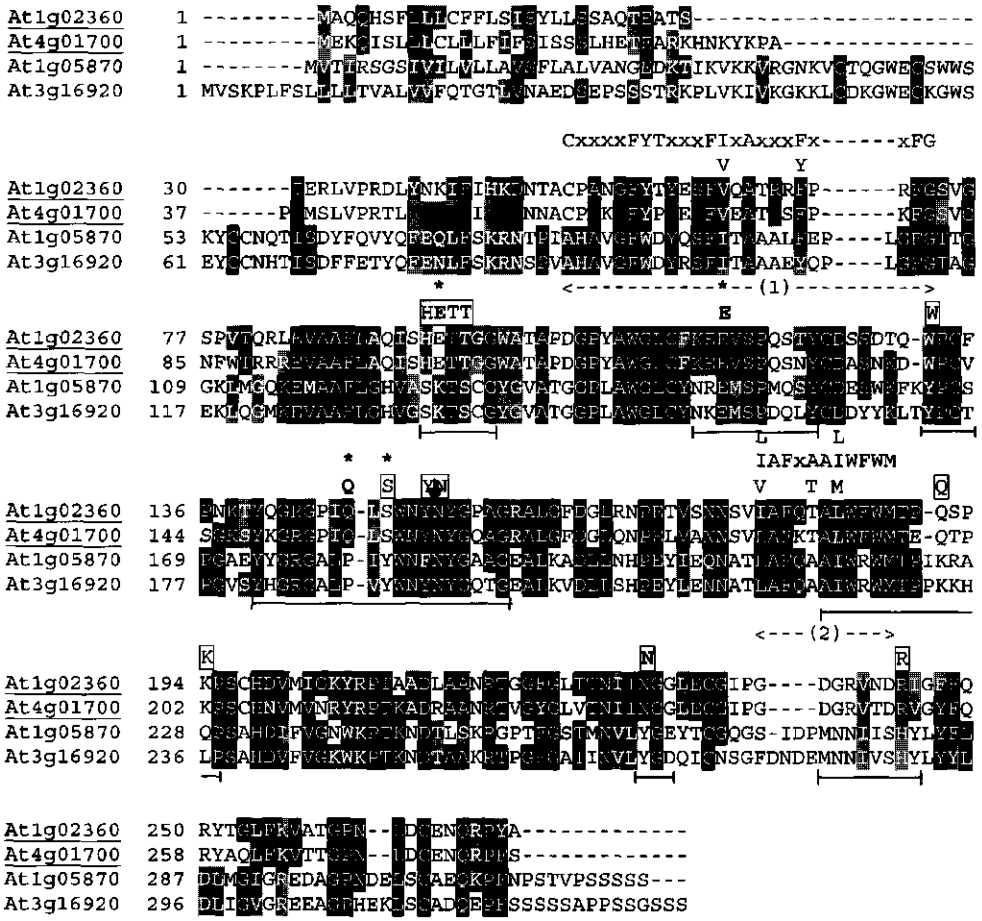


Figure 5. Multiple sequence alignement of *Arabidopsis* class II chitinases.
Gaps were introduced for optimal alignment and the degree of shading represents the level of similarity. PROSITE consensus patterns (Bairoch, 1992) are indicated above the aligned sequences and their names under. (1) stands 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.

As for class II chitinases, based on the missing essential amino acid residues and the failure to find ESTs we can conclude 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.

Figure 6. Multiple sequence alignement 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).

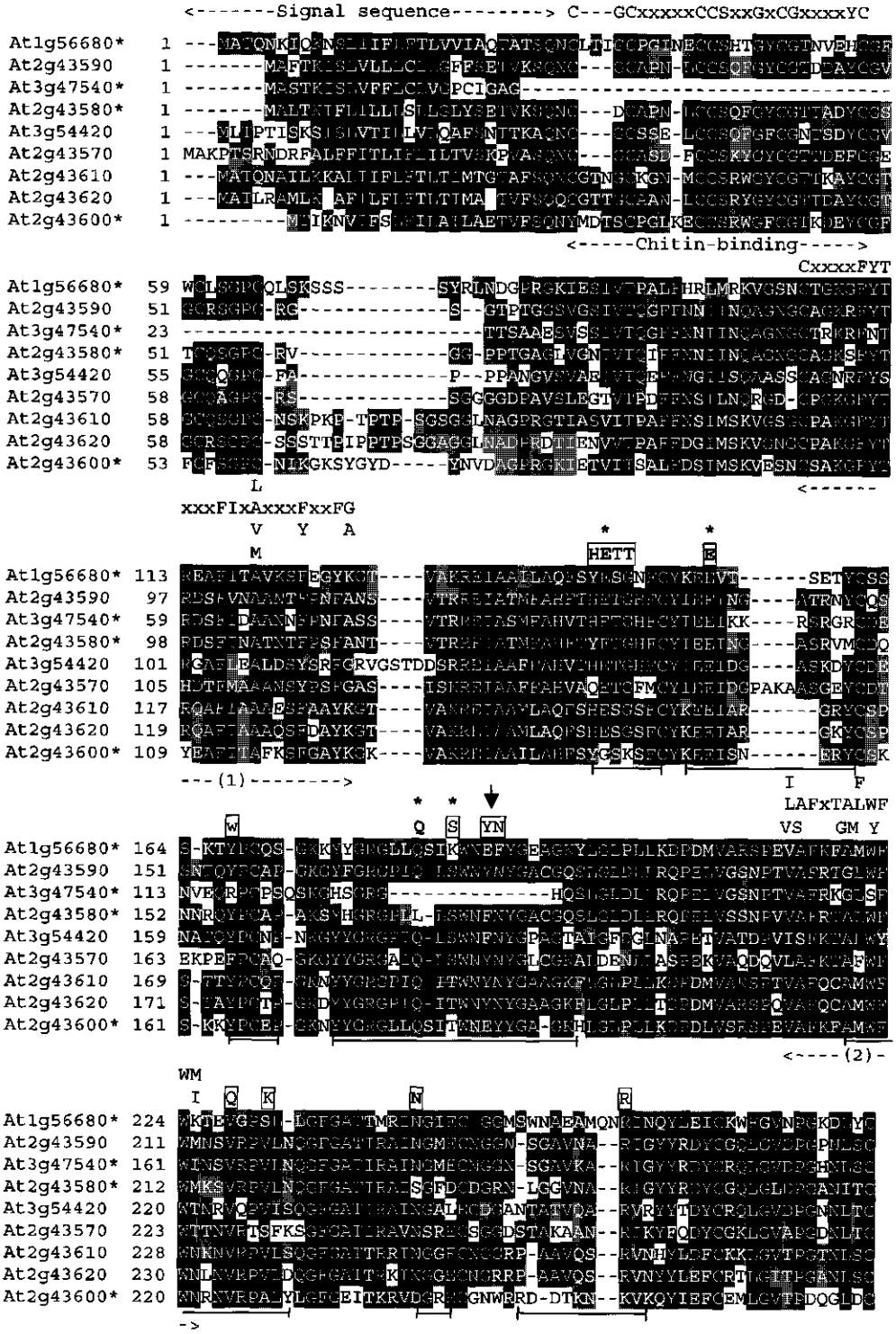

<-------Signal sequence--------> C---GCxxxxxCCSxxGxCGxxxxYC
At1g56680* 1
At2g43590 1
At3g47540* 1
At2g43580* 1
At3g54420 1
At2g43570 1
At2g43610 1
At2g43620 1
At2g43600* 1
<-----Chitin-binding----->
CxxxxFYT
At1g56680* 59
At2g43590 51
At3g47540* 23
At2g43580* 51
At3g54420 55
At2g43570 58
At2g43610 58
At2g43620 58
At2g43600* 53
xxxFIxAxxxFxxFG
At1g56680* 113
At2g43590 97
At3g47540* 59
At2g43580* 98
At3g54420 101
At2g43570 105
At2g43610 117
At2g43620 119
At2g43600* 109
---(1)-------->
LAFxTALWF
At1g56680* 164
At2g43590 151
At3g47540* 113
At2g43580* 152
At3g54420 159
At2g43570 163
At2g43610 169
At2g43620 171
At2g43600* 161
<----(2)-
WM
At1g56680* 224
At2g43590 211
At3g47540* 161
At2g43580* 212
At3g54420 220
At2g43570 223
At2g43610 228
At2g43620 230
At2g43600* 220
->

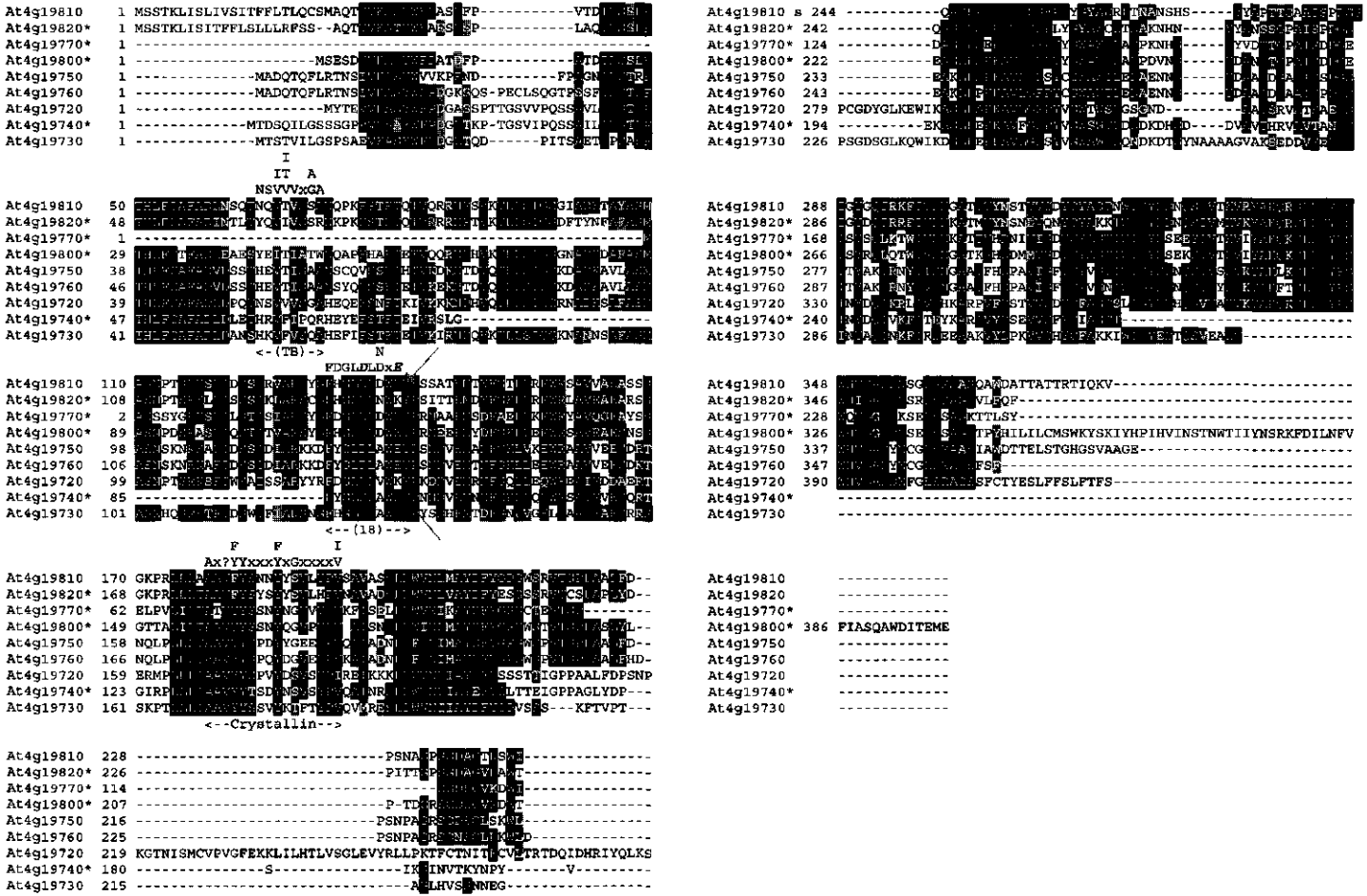
Figure 7. Multiple sequence alignement of *Arabidopsis* class V chitinases.
Gaps were introduced for optimal alignment and the degree of shading represents the level of similarity. The (*) marks the putative genes, for which no ESTs were found. PROSITE consensus patterns (Bairoch, 1992) are indicated 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)-[LIVMFCST]). 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).

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 targeted 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.


At4g19810
At4g19820*
At4g19770*
At4g19800*
At4g19750
At4g19760
At4g19720
At4g19740*
At4g19730
I
IT A
NSVVVxGA
<-(TB)-> N
FDGLDLDxE
<--(18)-->
F F I
Ax?YYxxxYxGxxxxV
<--Crystallin-->

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*; etc...	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); etc...	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) IPR000726 Chitinase family 19 (pfam00182 Glyco_hydro_19) 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); etc...	Putative gene (no ESTs found) encoding a protein similar to a probably inactive class IV chitinase.
At2g43570	IPR001002 Chitin-binding domain (pfam00187 chitin_binding) 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 plus seed chitinase A PIR: P29022 from *Zea mays* (antifungal role; (Huynh *et al.*, 1992)); etc...	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) IPR000726 Chitinase family 19 (pfam00182 Glyco_hydro_19; PS00773 CHITINASE 19_1 and PS00774 CHITINASE 19_2) 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)); etc...	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) 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)); etc...	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) 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 BAA22985.1 from *Chenopodium amaranticolor*; etc...	Putative gene (no EST found) encoding a probably inactive secreted class IV chitinase. Unknown function.

Table 2. Characteristics and reannotation of the *Arabidopsis* chitinase genes (continued).
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 "probable signal sequence" 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.

Locus	InterPro domain search	Targeting	Similarities	Reannotation - Remarks
At2g43610	IPR000726 Chitinase family 19 (pfam00182 Glyco_hydro_19) IPR001002 Chitin-binding domain (pfam00187 chitin_binding and PS00026 CHITIN_BINDING)	PSORT: outside 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*; etc...	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) IPR001002 Chitin-binding domain (pfam00187 chitin_binding and PS00026 CHITIN_BINDING)	PSORT: outside 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*; etc...	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) IPR001002 Chitin-binding domain (pfam00187 chitin_binding and PS00026 CHITIN_BINDING)	PSORT: outside 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)); etc...	Transcribed sequence encoding a vacuolar active class I chitinase (not compatible with computer-proposed targeting). Developmentally regulated, possibly involved in pathogen responses and senescence, linked to ethylene signaling.
At3g16920	IPR000726 Chitinase family 19 (pfam00182 Glyco_hydro_19)	PSORT: outside 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)); etc...	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 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); etc...	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) IPR001002 Chitin-binding domain (pfam00187 chitin_binding and PS00026 CHITIN_BINDING)	PSORT: outside 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)); etc...	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 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; etc...	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) IPR000531 TonB-dependent receptor protein (PS00430 TONB-DEPENDENT_REC1) 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)); etc...	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.

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

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) IPR001064 Crystallin (PS00225 CRYSTALLIN_BETAGAMMA) 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) IPR001064 Crystallin (PS00225 CRYSTALLIN_BETAGAMMA) 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) IPR001579 Chitinases family 18 and 2 (PS01095 CHITINASE_18) 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) 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 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) 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 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)); etc...	Transcribed sequence encoding an active secreted class III chitinase, possibly involved in response to specific pathogens.

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

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::β-glucuronidase (GUS) studies, the class III chitinase is expressed in roots, leaf vascular tissue, 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 functions, 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 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

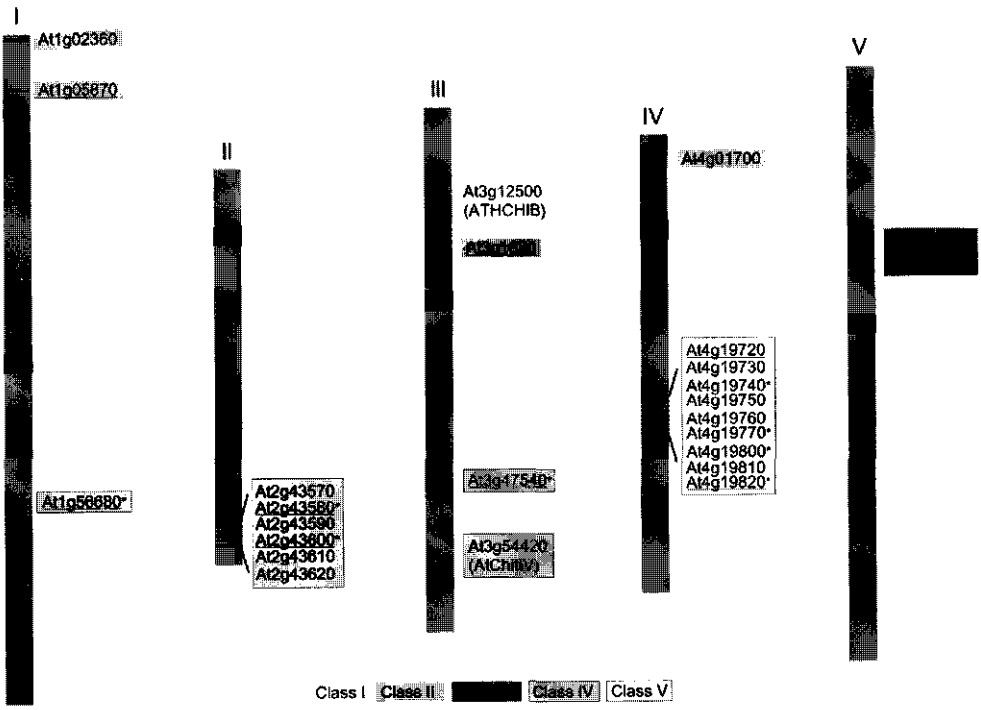


Figure 8. Recapitulation of the characteristics of the *Arabidopsis* chitinase annotations.
As in Figure 1, the locus of each annotation is indicated on the five *Arabidopsis* chromosomes. The (*) indicates sequences that are not likely to be transcribed. The degree of shading and the boxes around the 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.

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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Introduction

Somatic embryos have been used extensively to identify genes involved in embryogenesis, while the conditioned medium of embryogenic cultures is a rich source of endogenous molecules promoting the formation of somatic embryos (de Vries *et al.*, 1988; Maës *et al.*, 1997; Schmidt *et al.*, 1994). The mutant carrot cell line *ts11*, impaired in embryogenesis, allowed the identification of one of these molecules. The *ts11* mutant only forms somatic embryos at permissive temperature (Lo Schiavo *et al.*, 1990) while at non permissive temperature mutant embryos require medium conditioned by wild-type cells in order to develop beyond the globular stage. The component in the conditioned medium responsible for lifting the arrest was purified and identified as an acidic endochitinase of 32 kDa, designated as EP3 (de Jong *et al.*, 1992). The EP3 chitinase not only promoted the globular to heart stage transition, but also the formation of globular embryos (de Jong *et al.*, 1993). Because in *ts11* media a fully active EP3 chitinase was present, it was concluded that *ts11* does not have a structural mutation in the encoding gene. Instead, it was found that the secretion of EP3 appeared to be transiently reduced in *ts11* during the early globular stage (de Jong *et al.*, 1995). The ability to rescue *ts11* embryos was also confined to this same period. These results suggested a specific and a transient role of the chitinase during somatic embryogenesis.

Molecular cloning of the gene encoding the carrot EP3 chitinase revealed that the EP3 protein belongs to the class IV chitinase family of which 4 members have been cloned (Kragh *et al.*, 1996). At least 5 different EP3 isoenzymes were shown to be present in the conditioned medium of wild-type cultures (Kragh *et al.*, 1996). Three of these EP3 isoenzymes, as well as a related class I chitinase, showed *ts11* embryo rescue activity, but exhibited subtle differences in their biological effect (Kragh *et al.*, 1996). The existence of multiple EP3 isoenzymes encoded by a small multigene family supported the findings that *ts11* does not have a structural mutation in a single chitinase gene, but is affected in the control of the extracellular level of several secreted chitinases. The effect of the chitinases was mimicked by Nod factors and it was therefore proposed that these chitinases are involved in the generation of signal molecules essential for somatic embryogenesis in *ts11* (de Jong *et al.*, 1993).

The *EP3* genes were shown to be expressed in a subset of most likely non-embryogenic carrot suspension cells. In planta, the EP3 genes were expressed at low level throughout the plant, but highest during seed development. In situ mRNA localization revealed that EP3 gene expression was highest in seeds 10 days after pollination (DAP), and found predominantly in the inner integument cells lining the embryo sac. Later, expression

was restricted to a small subset of endosperm cells lining the central cavity in which the embryo develops. These results were in line with the hypothesis that the EP3 chitinases could have a "nursing" function during zygotic embryogenesis and that this function can be mimicked by some of the suspension cells during somatic embryogenesis (van Hengel *et al.*, 1998).

Other plant chitinases were found to be expressed during seed development. Northern analysis showed that in soybean a chitinase was expressed in developing seeds (Yeboah *et al.*, 1998). In barley, several chitinases were shown by immunoblotting, in situ hybridization and GUS immunolocalization to be expressed in the aleurone, the endosperm and the embryo (Leah *et al.*, 1994; Swegle *et al.*, 1992), suggesting their involvement during embryogenesis and seed development.

Based on sequence homology, immunological relationship and biochemical activity we have identified and cloned the *Arabidopsis* ortholog of the carrot *EP3* gene. As in carrot, the *Arabidopsis* gene is not expressed in somatic embryos, but in cells of embryogenic clusters during somatic embryogenesis. In plants, the *AtEP3* gene is expressed in germinating pollen and growing pollen tubes, and not in endosperm and integuments as in carrot. Later, the *AtEP3* gene is expressed in the root epidermis, hydathodes and stipules.

Results

The Arabidopsis ortholog of the carrot EP3 genes

Searching the *Arabidopsis* genome with the carrot EP3 sequence revealed the EST *tai224* (GenBank Z26409) as the most likely candidate. Although, *tai224* shared only 59.2% identity with EP3 at the amino acid level, but with 95% of the *Arabidopsis* genome sequencing completed, no chitinase gene was found with higher homology. Yet, class IV chitinases in *Arabidopsis* belong to a small multigene family with six other putative gene members (*At2g43570, At2g43580, At2g43590, At2g43600, At2g43610* and *At2g43620*) found in tandem on a BAC of chromosome II (GenBank AC002333). Nevertheless they share lower identities at the amino acid level, ranging from 34.4 to 50.2% and important domains of the proteins are less conserved than between tai224 and EP3 (Figure1). A corresponding genomic clone *λAtEP3* was obtained and found to be identical to *AtchitIV* (GenBank Y14590; de A. Gerhardt *et al.*, 1997). The full length *AtEP3* cDNA has an open reading frame of 822 bp, corresponding to an acidic protein of 273 amino acids with a predicted molecular weight of

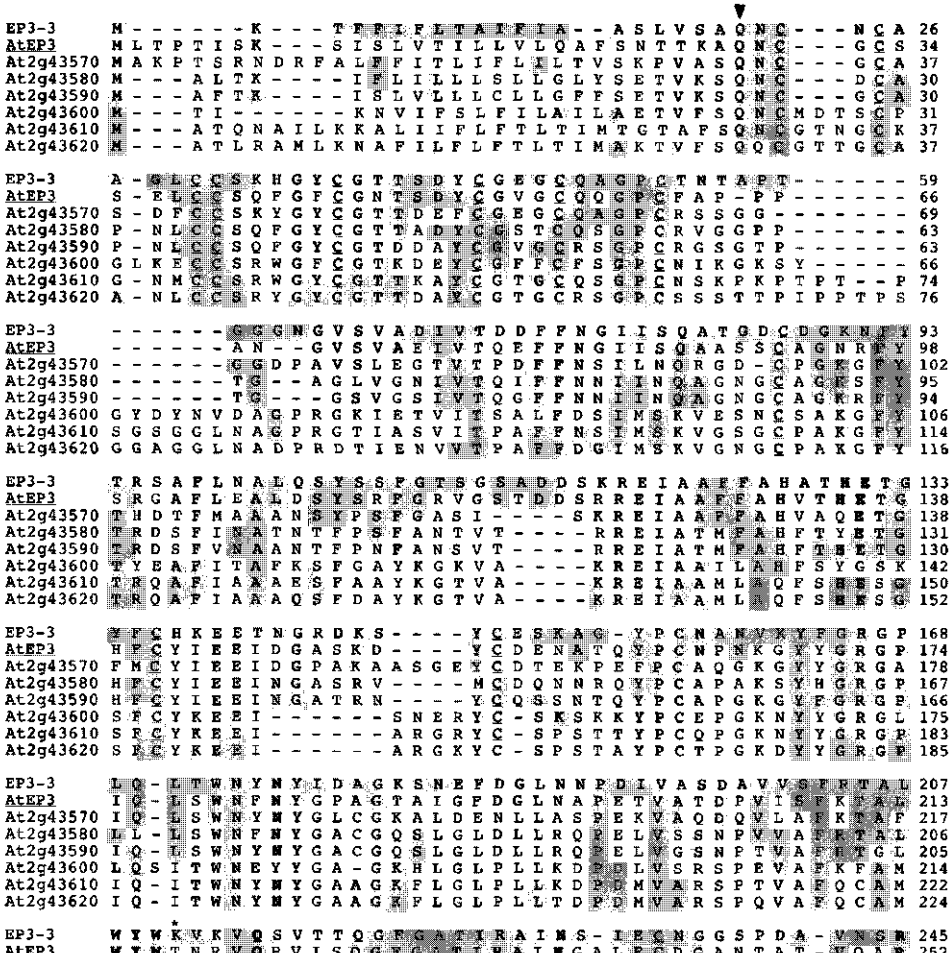

Figure 1. Comparison of the deduced amino acid sequence of AtEP3 and other *Arabidopsis* class IV chitinases with the carrot EP3 chitinase.

Sequences are deduced from the corresponding cDNAs, EP3-3 being the carrot class IV chitinase (de Jong *et al.*, 1992). AtEP3 is underlined. Gaps were introduced for optimal alignment. The EP3-3 sequence and the residues identical to this sequence are shaded. Predicted signal sequence cleavage site is shown by the arrowhead. Cysteine residues forming disulfide bonds are underlined. Residues postulated to be involved in substrate binding and catalysis are shown in bold. And position of a conserved methionine among chitinases of this class but absent from EP3 and AtEP3 is shown by *

29.4 kDa. When compared to the EP3-3 sequence of carrot (Figure 1), the AtEP3/AtchitIV amino acid sequence shows all characteristics of the EP3 class IV chitinases. The predicted amino acid sequence has a 28 amino acid signal sequence, probably cleaved before the glutamate residue (arrow at position 29 of the AtEP3 sequence in Figure 1). The N-terminus of the mature protein then commences with a cysteine-rich region, between the positions 29 and 59 that is assumed to be the substrate-binding domain. Next there is the same short hinge region as in the carrot EP3, between residues 60 and 74, followed by the catalytic domain between residues 75 and 273 (Collinge *et al.*, 1993). Except for the signal sequence, each of these domains is reasonably conserved. All cysteines are conserved (underlined in Figure 1), as well as all residues assumed to be involved in catalysis (in bold in Figure 1; Andersen *et al.*, 1997; Verburg *et al.*, 1993). Like all EP3 isoenzymes (Kragh *et al.*, 1996), AtEP3/AtchitIV does not contain any methionine in the mature protein, while other known class IV chitinases contain one conserved methionine at position 217 (Figure 1).

To determine whether an *Arabidopsis* EP3 chitinase was secreted into the medium of an embryogenic culture from *Arabidopsis*, a Western blot of secreted proteins was probed with antiserum raised against the carrot EP3 endochitinase (Kragh *et al.*, 1996). Whereas in carrot medium five isoforms were detected (Kragh *et al.*, 1996 and Figure 2A), only a single protein was recognized by the antiserum in the *Arabidopsis* medium (Figure 2A). This indicates that there might be a single ortholog of EP3 in the *Arabidopsis* genome, although seven different class IV chitinase genes were found so far. To demonstrate that the *AtEP3/AtchitIV* gene encodes the secreted AtEP3 protein as recognized by the carrot EP3 antiserum (Figure 2A), the full length *AtEP3* cDNA was introduced into Sf21 insect cells using the baculovirus-based expression system. The medium of AtEP3-producing Sf21 cells contained a single protein that cross-reacted with heterologous antisera raised against carrot and sugar beet class IV chitinases (Figure 2B).

In contrast to carrot, where the EP3 endochitinase was found in roughly equivalent amounts in both embryogenic and non-embryogenic cell cultures (Kragh *et al.*, 1996; van Hengel *et al.*, 1998), AtEP3 is produced in embryogenic suspension cultures only (Figure 2B). The AtEP3 protein was purified from insect cell cultures and compared with the native AtEP3 purified from *Arabidopsis* cultures as well as with the carrot EP3-3 protein similarly produced in Sf21 insect cells. Using ^{3}H-chitin as substrate, the results show a comparable specific activity and pH optimum for all 3 chitinases (Table 1). The lower specific activity of the Sf21-produced AtEP3 compared to the native chitinase could be due to an incorrectly folded or less

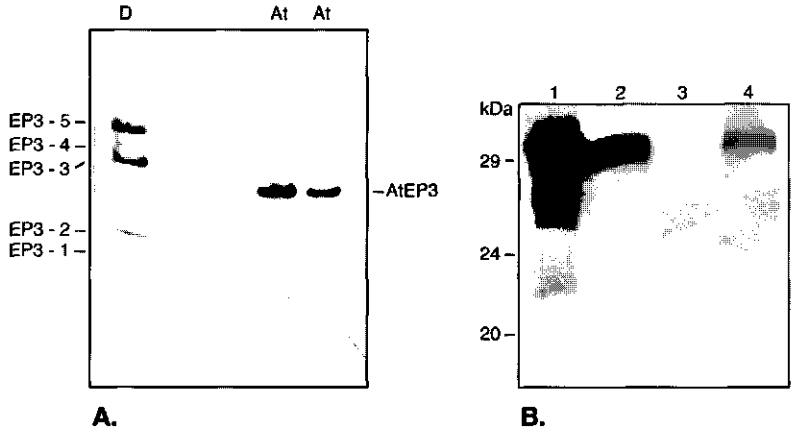

Figure 2. Immunological relationship between AtEP3/AtchitIV and the carrot EP3 chitinase.
A. Western blot produced on proteins from medium of *Daucus* cell suspension cultures (lane D), and of *Arabidopsis* embryogenic cultures (lanes At), incubated with an antiserum raised against the carrot EP3 chitinase. EP3-1 to EP3-5 represent the different isoforms of the carrot chitinase and AtEP3 the corresponding *Arabidopsis* chitinase. **B.** Western blot produced on proteins from: lane 1. Sf21 insect cell medium where AtEP3 is expressed using the baculovirus-based expression system; lane 2. AtEP3 protein purified from the insect cell medium; lane 3. *Arabidopsis* non-embryogenic culture medium; lane 4. *Arabidopsis* embryogenic culture medium. The Western blot was incubated with antisera raised against carrot and sugar beat class IV chitinases (de Jong *et al.*, 1992; Kragh *et al.*, 1996).

stable enzyme. An altered state of the Sf21-produced proteins is also suggested by the results obtained while testing the purified enzymes in a *ts11* embryo rescue assay. The assay was not successful for any of the two Sf21 cell-produced chitinases, whereas the native *Arabidopsis* chitinase was able to rescue *ts11* embryos (Table 1). Nevertheless, based on the above criteria, we conclude that AtEP3/AtChitIV is the *Arabidopsis* ortholog of the carrot EP3 gene family.

	Specific activity nmol GlcNAc min^{-1} mg^{-1}	pH optimum	ts11 rescue
EP3-3 native	nd	nd	+
EP3-3 Sf21	3200	5.0	-
AtEP3 native	6000	4.8	+
AtEP3 Sf21	4200	5.0	-

Table 1. Biochemical relationship between AtEP3/AtchitIV and the carrot EP3 chitinase.
EP3-3 and AtEP3 chitinases were purified from embryogenic culture medium (EP3-3 native, AtEP3 native) and from Sf21 insect cell medium (EP3-3 Sf21, AtEP3 Sf21) where they were expressed using the baculovirus-based expression system. Specific activity and pH optimum were determined using ^{3}H-chitin as substrate. The *ts11* rescue assay was carried out as described in de Jong *et al.* (1992). nd = not determined; + = rescue; - = no rescue.

Mapping of AtEP3/AtchitIV

Mapping of *AtEP3/AtchitIV* was performed using 101 Recombinant Inbred (RI) lines (Lister and Dean 1993) and a *Dra*I RFLP between the ecotypes Landsberg *erecta* (Ler) and Columbia (Col) (Figure 3A). The *AtEP3/AtchitIV* gene is located at the bottom of chromosome 3, 9.4 cM below the *TSA1* gene (see the RI map released at http://nasc.nott.ac.uk/new_ri_map.html, where for *AtEP3/AtchitIV* read *tai224*). *AtEP3/AtchitIV* was also physically mapped by: (i) Fluorescence In Situ Hybridization (FISH) on pachytene chromosomes (Figure 3B); and (ii) hybridization to the CIC YAC library (Creusot *et al.*, 1995) which showed that, like *TSA1*, *AtEP3/AtchitIV* is in fact located higher on the lower arm of chromosome 3 (Figure 3C). This also revealed a discrepancy between the genetic and physical maps in this region of chromosome 3. No known mutation has so far been identified in the vicinity of the *AtEP3/AtchitIV* locus (see the classical genetic map of *Arabidopsis* at http://mutant.lse.okstate.edu/).

The hybridization pattern seen in Figure 3A, at the stringency used (2xSSC at 65°C), suggests that *AtEP3/AtchitIV* is a single copy gene. The *Hind*III digestion for instance gives a single hybridizing band of 1 kb corresponding to the *Hind*III-*Hind*III fragment within the coding sequence (Figure 3D). The completed sequencing of chromosome 3 in this region allowed verifying the validity of the rest of the hybridization pattern, ruling out the possibility of another copy of the gene nearby. The physical mapping (YAC hybridization and FISH) supports the Southern blot analysis showing that the *AtEP3/AtchitIV* gene is a single copy gene, as opposed to the two genes proposed previously (de A. Gerhardt *et al.*, 1997).

Expression of the AtEP3/AtchitIV gene

Analysis by RT-PCR

The *AtEP3/AtchitIV* mRNA was not detectable by Northern analysis on 10 µg of total RNA from either leaves, roots or siliques (data not shown). RT-PCR was then performed using *AtEP3/AtchitIV* gene-specific primers on reverse-transcribed cDNA from total RNA of flowers, stems, roots, old and young siliques, and seedlings. Agarose gel blot analysis was carried out on the PCR products using the radiolabeled *AtEP3/AtchitIV* cDNA as a probe. Transcripts were detected in all the tissues analyzed as a hybridizing band of 822bp. The highest level of expression was found in seedlings (Figure 4A). A control RT-PCR was performed on the same reverse-transcribed cDNA using ubiquitin gene-specific primers. Agarose gel analysis of the PCR products shows that the reverse transcribed cDNA amounts

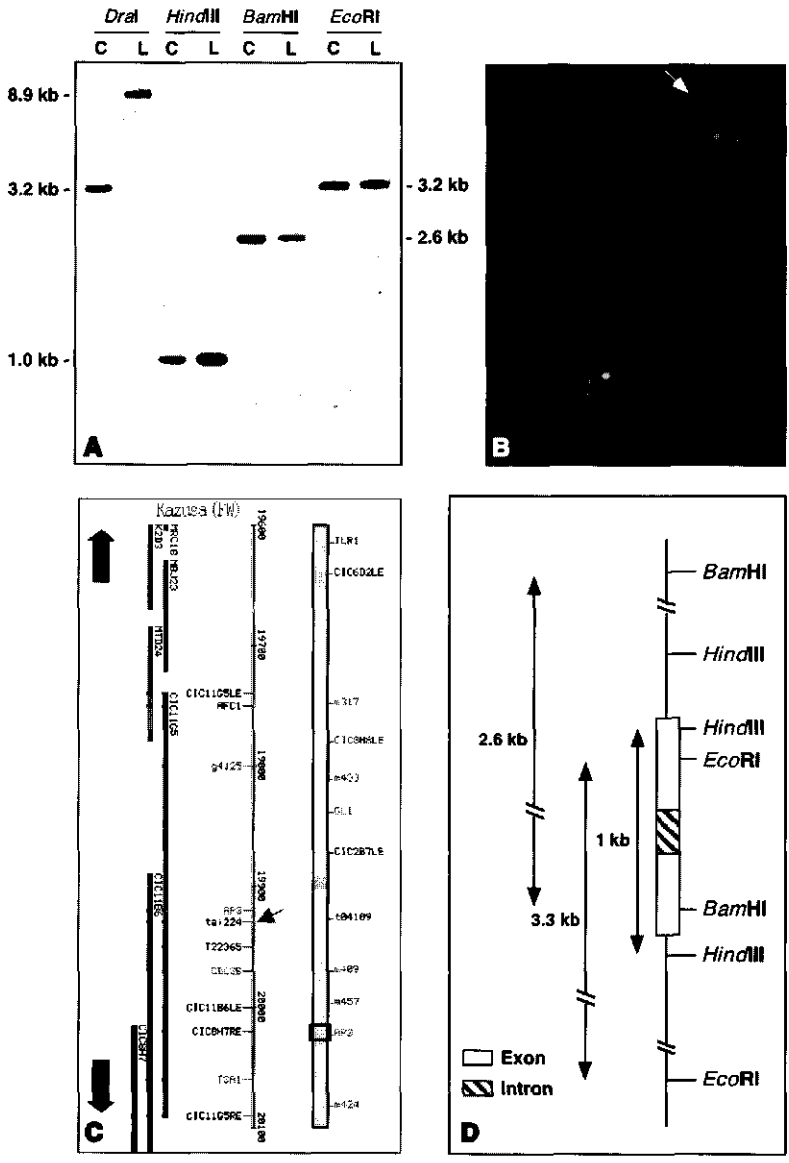

Figure 3. Genetic and physical mapping of the *AtEP3/ AtchitIV* gene.
A. Genomic Southern blot from RIL parental DNA (Columbia (C) and Landsberg *erecta* (L)) hybridized with the *AtEP3/AtchitIV* cDNA probe. RFLP used for the mapping was revealed by the enzyme *Dra*I (first 2 lanes). **B.** Fluorescence In Situ Hybridization mapping. Superimposition picture of DAPI stained pachytene chromosomes and hybridization signals. The green signal indicated by the arrow represents the *AtEP3/AtchitIV* locus on the lower arm of chromosome 3, whereas the red signals represent 5S rDNA located on the short arm of chromosome 4 and on the upper arm of chromosome 5. **C.** Physical map of chromosome 3. The *AtEP3/AtchitIV* locus is indicated by the red arrow and named after the EST probe used for the mapping, tai224. The physical map depicted here was obtained from the *Arabidopsis thaliana* Database, at http://genome-www3.stanford.edu/cgi-bin/AtDB/Pmap. **D.** Restriction map of the *AtEP3/AtchitIV* locus.

used as template were comparable in each sample (Figure 4B). These results are in line with those found with the carrot EP3 genes, for which expression is also observed in other plant organs, although predominantly in developing seeds (van Hengel *et al.*, 1998).

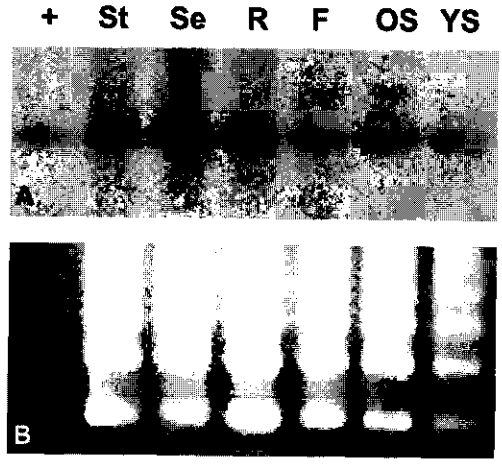

Figure 4. Analysis by RT-PCR of *AtEP3/AtchitIV* expression in *Arabidopsis* plants.
The RT-PCR was performed on total RNA from stems (St), seedlings (Se), roots (R), flowers (F), old siliques (OS) and young siliques (YS). The positive control for the PCR reaction (+) was 10 pg of plasmid containing the *AtEP3/AtchitIV* cDNA. **A.** Autoradiogram of the PCR products obtained using AtEP3/AtchitIVgene-specific primers after hybridization with the *AtEP3/AtchitIV* cDNA. **B.** Agarose gel of the PCR products obtained using ubiquitin gene-specific primers.

Promoter::reporter expression

Expression of the *AtEP3/AtchitIV* gene was monitored in detail by the use of a promoter::*GUS* construct transformed into *Arabidopsis*. Eleven independent transformants were obtained for the *AtEP3/AtchitIV::GUS* construct and their progeny allowed to self. Plants of each T_2 line were stained for expression of the *GUS* reporter gene. All lines showed identical expression patterns differing only in staining intensity. Two of the strongest expressing lines were therefore chosen for detailed analysis on homozygous T_3 plants. Plants were transformed in parallel with an *AtEP3/AtchitIV*promoter::luciferase (LUC) construct. The progeny of two independent transformants was assayed for luciferase activity. No change in expression was found when compared with the GUS data (see Figure 6A, B and 7H, M).
Because the role of the carrot EP3 during embryogenesis was originally demonstrated during somatic embryogenesis, it was important to verify whether the *AtEP3/AtchitIV* gene is similarly expressed in *Arabidopsis*. Embryogenic and non-embryogenic cell suspension cultures of *Arabidopsis* can be established from wild-type immature zygotic embryos or directly from germinating seeds of the mutant *primordia timing* (*pt*, Mordhorst *et al.*, 1998).

AtEP3/AtchitIV::GUS-expressing embryogenic cultures were initiated from isolated immature zygotic embryos of the two homozygous promoter::GUS lines. In these lines *GUS* expression was found to be similar to that of the carrot *EP3* genes (van Hengel *et al.*, 1998). The *AtEP3/AtchitIV* gene appeared to be expressed in cells close to the developing embryos but not in the embryos themselves. Promoter activity is observed after overnight staining in embryogenic clusters and never in the embryo at any stage (Figure 5A). In contrast with the carrot EP3 genes (van Hengel *et al.*, 1998), *AtEP3/AtchitIV* expression is restricted to embryogenic cultures and absent from yellowish non-embryogenic clusters or cultures (Figure 5B). These results confirmed the Western blot analysis, showing that the AtEP3/AtchitIV chitinase was only detectable in the medium of embryogenic cultures (Figure 2B).

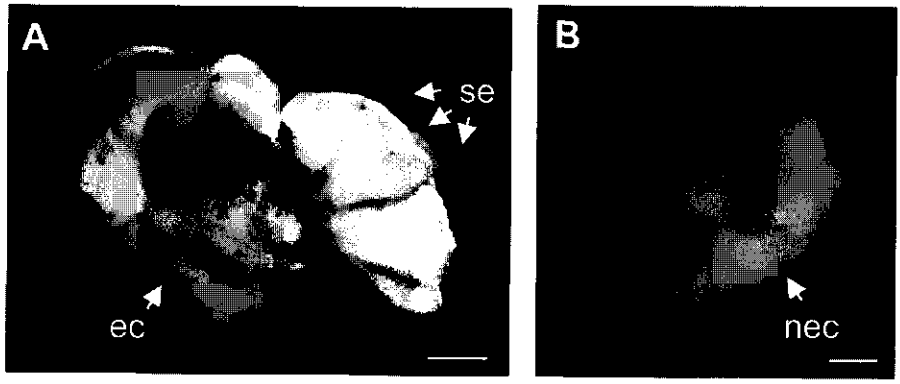

Figure 5. Histochemical localization of GUS activity during somatic embryogenesis in *Arabidopsis* cultures containing the *AtEP3/AtchitIV* promoter::GUS fusion.
Embryogenic cluster (**A**) and non-embryogenic cluster (**B**) from *Arabidopsis* embryogenic lines initiated via dissection of immature zygotic embryos according to Mordhorst *et al.*, (1998). ec, embryogenic cluster; nec, non-embryogenic cluster; se, somatic embryo. Bar = 1 mm.

In flowering plants, *GUS* expression was observed after 2 to 5 days of staining, in a stage dependent manner in pollen. Activity of GUS is absent from young developing pollen, and increases during pollen maturation (Figure 6A), as was also found when measuring luciferase activity under the control of the *AtEP3/AtchitIV* promoter (Figure 6B). GUS expression persists after pollen germination in the pollen tubes growing along the placenta and the funiculus (Figure 6C-E). Upon fertilization the GUS-expressing pollen tube enters the receptive synergid via the micropyle in order to release the two sperm cells in the embryo sac. As this occurs, both receptive synergid and central cell turn blue (Figure 6F). While the

zygote develops, GUS product remains in the degenerating receptive synergid (Figure 6G) and in some rare cases in the free nuclear endosperm as well (Figure 6H). To determine whether the staining in the receptive synergid and central cell represents embryo sac gene expression or release of the GUS product from the bursting pollen tube, *AtEP3/AtchitIV::GUS* plants were pollinated with wild-type pollen and vice-versa. It appeared that GUS expression in the embryo sac was never observed in the case of fertilization with wild-type pollen (Figure 6I), but only in ovules (wild-type or *AtEP3/AtchitIV::GUS*) fertilized with *AtEP3/AtchitIV::GUS* pollen. This indicates that the GUS product present in the embryo sac is released by the pollen tube. Thus, some constituents of the male gametophyte including the AtEP3/AtchitIV chitinase can be transferred along with the sperm cells into the embryo sac. Expression was not seen during embryo development up to the mature stage (Figure 6J), except in malformed and aborted seeds, in which the embryos appear to be misshapen (Figure 6 K). Expression of GUS reappears during germination in the differentiating root-hypocotyl transition zone of the young germinating seedling (Figure 7A and B). It is also visible in some endosperm or seed coat cells at the place where the radicle protrudes (Figure 7B and C). The cytoplasm of the cells where the staining is localized seems to show signs of shrinkage suggesting that these cells are dying. As the seedling develops, the transition zone differentiates, and the first root hair initials and root hairs appear, accompanied by GUS expression in some cells of this area (Figure 7B and D). At higher magnification, the mosaic-like staining pattern appears to represent cells that are differentiating into root hair initials (Figure 7E, arrowheads). Further development of the seedling confirms the nature of this pattern (Figure 7F and G), clearly showing GUS activity in elongating root hairs (Figure 7G and H). Moreover the staining seems confined to the epidermis layer (Figure 7G) and extends as the specialization zone does. Staining one week-old seedlings for GUS shows indeed that expression remains in the root epidermis of the adult root specialization zone (Figure 7H) and is absent from the elongation zone (Figure 7I). In the aerial parts of the plant, expression was restricted to the few cells forming the stipules and the hydathodes (Figure 7H and J-L), all of which is also found in *AtEP3/AtchitIV::LUC* seedlings (Figure 7M). Hydathodes are known as entrance points for pathogens (Hugouvieux *et al.*, 1998) and roots are also exposed to numerous sources of pathogens. Therefore, in order to check for a possible pathogen or stress induction of the *AtEP3/AtchitIV* gene, plants were grown in non- sterilized soil prior to GUS staining. No change in staining pattern or intensity was observed when compared to plants grown in vitro (data not shown), confirming the lack of pathogen inducibility of the carrot EP3 gene (van Hengel *et al.*, 1998).

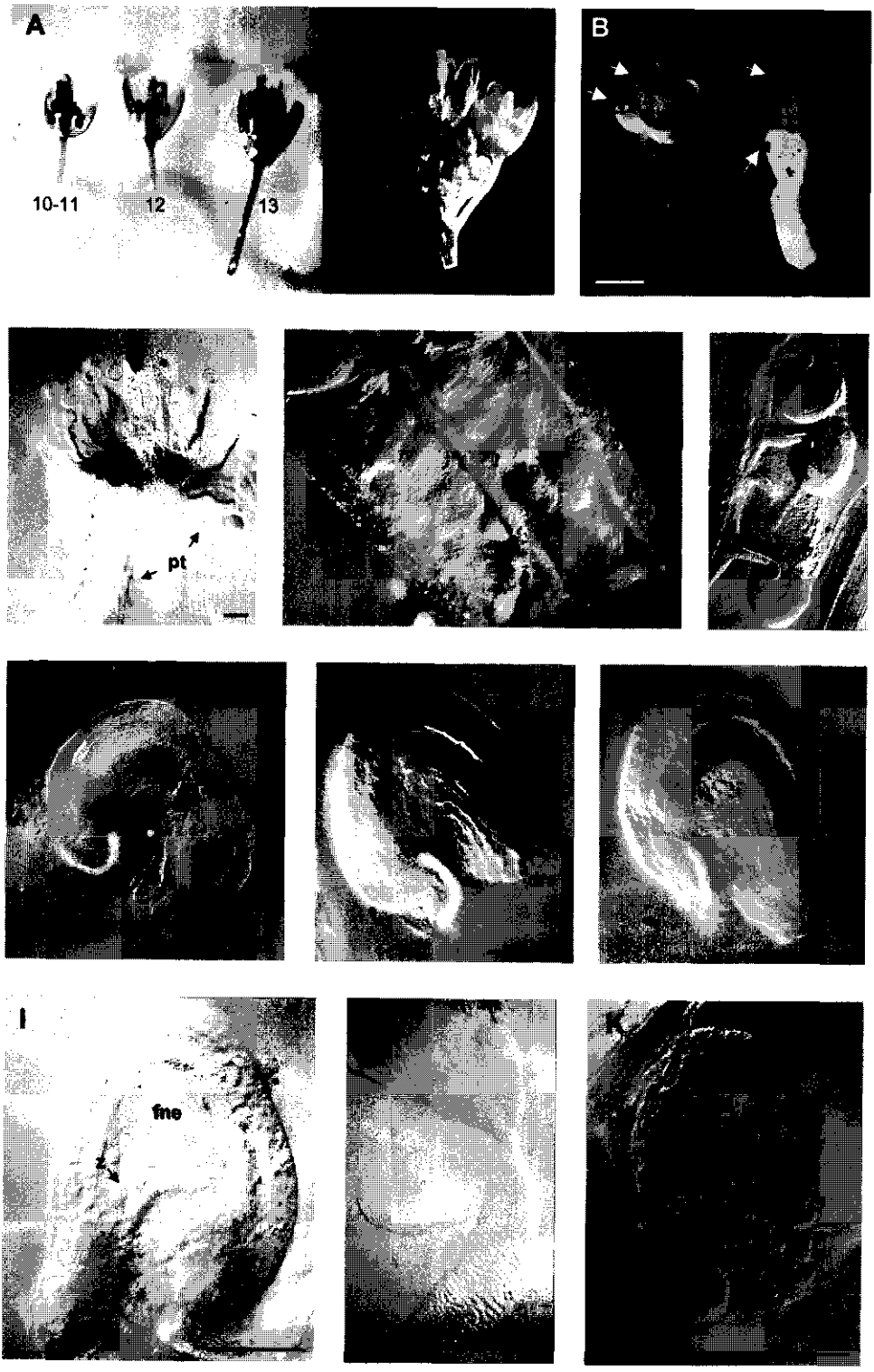
A
10-11
12
13
B
pt
I
fne

Figure 6. Localization of *AtEP3/AtchitIV* promoter::reporter activity in *Arabidopsis* flowering plants.
A. Developing flowers from stage 10-11 to 13 and a complete inflorescence stained for GUS, showing the stage-dependent expression of the reporter gene. **B.** Luciferase activity measured in flowers from stage 14 and 15. Photons are emitted by single pollen grains (arrows). **C-D.** Expression of GUS in germinating pollen on the surface of the stigma and growing pollen tubes (pt). **E.** Expression of GUS in pollen tubes during entry trough the micropyle. **F.** Ovule just after fertilization with GUS expression in the receptive synergid (rs) and the central cell (cc). **G.** Seed with developing zygote (z) and GUS expression in degenerating receptive synergid (drs). **H.** Young developing seed with GUS expression in the free nuclear endopserm (fne) and the degenerating receptive synergid (drs). **I.** Developing seed from an *AtEP3/AtchitIV::GUS* flower pollinated with wild-type pollen. No GUS expression is observed in the embryo sac. **J.** Mature embryo stained for GUS, popped out of a seed prior to germination. **K.** Aborted seed with GUS expression in the arrested embryo. All other seeds from the same silique were already mature. Bar = 50 μm.

The expression patterns described here were obtained on 11 independent promoter::*GUS* transformants and confirmed by the use of the luciferase reporter gene, although luciferase activity measurements do not allow a detailed analysis at the cell level. They also confirm the results of the RT-PCR analysis at the organ level. Despite numerous attempts, none of these results could be confirmed at the cellular level by in situ hybridization on sections or in whole mounts. This is most likely due to low steady state mRNA levels, only detectable by RT-PCR.

Discussion

The aim of the present work was to identify and characterize an *Arabidopsis* ortholog of the carrot *EP3* chitinase. Our results show that the *AtEP3/AtchitIV* gene is the most likely candidate. We base this on (i) sequence homology, (ii) immunological cross-reactivity of the encoded proteins, (iii) biochemical activity and (iv) somatic embryo rescue activity. As was found in carrot (Kragh *et al.*, 1996), in *Arabidopsis* there exists a small multigene family of related genes. When comparing expression of the *AtEP3/AtchitIV* gene with that of the carrot EP3 gene, correspondences as well as differences were observed. It appears for instance that the overall level expression in *Arabidopsis* is lower.

In embyogenic cell cultures of *Arabidopsis*, expression was restricted to a small subpopulation of single cells and cell clusters, and not in the embryo itself. This confirms the hypothesis made by van Hengel *et al.* (1998) of a possible "nursing" function during embryogenesis. The *Arabidopsis* gene is however only expressed in embryogenic cultures whereas the carrot genes are expressed in non-embryogenic cultures as well. This could be the result of the simultaneous detection of several members of the carrot gene family by RT-PCR

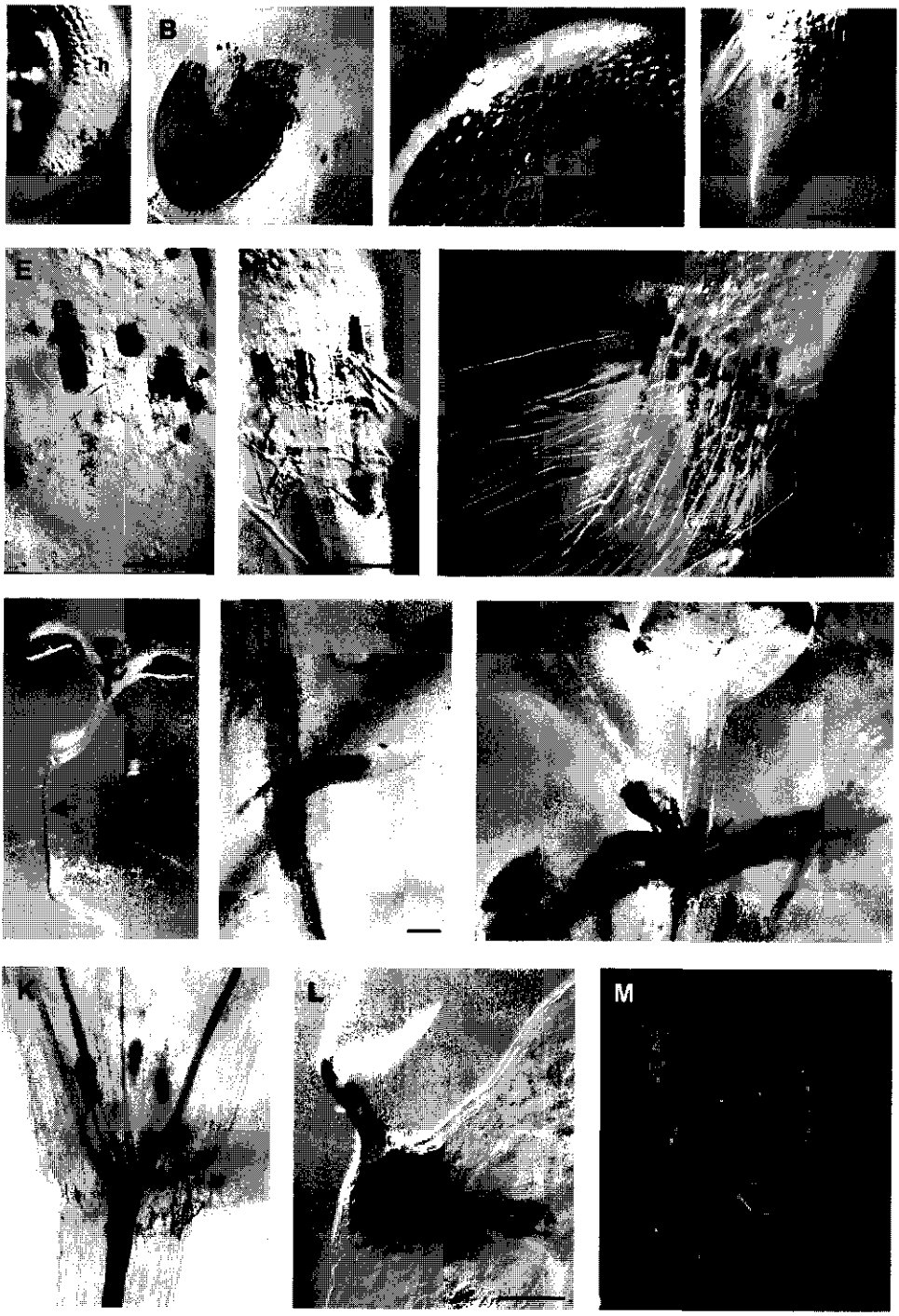
B
E
K
L
M

Figure 7. Histochemical localization of GUS activity in *Arabidopsis* germinating and developing seedlings containing the *AtEP3/AtchitIV* promoter::GUS. A. Detail of a protruding radicle, with the embyonic root (r), the hypocotyl (h) and initiation of the root-hypocotyl transition zone (t) where expression of GUS is observed in some cells. **B.** Protruding embryo. The root-hypocotyl transition zone is already well defined. The first root-hairs have developed and the number of cells expressing GUS increased. The cotyledons are still within the seed and the disrupted seed coat expressing GUS is visible. **C.** Detail of the disrupted seed coat upon germination observed in B. **D.** Detail of the root-hypocotyl transition zone observed in B. **E.** Higher magnification of a root-hypocotyl transition zone. Some root hair initials (arrowheads) and epidermal cells are expressing GUS. **F.** Detail of the specialization zone of a further developed seedling. **G.** Expression of GUS in elongating root-hairs and epidermal cells of the specialization zone. **H.** One week-old seedling. Root expressing GUS, stipules and hydathodes are indicated by the arrows. **I.** Higher magnification of the root, and of a lateral root, with no expression of GUS in the elongation zone. **J.** Shoot meristem area. Stipules and hydathodes expressing GUS are indicated by the arrows. **K.** Higher magnification of the shoot meristem area, showing expression of GUS in the stipules. **L.** Microscopic observation under Nomarski optics of an hydathode expressing the GUS gene. **M.** Luciferase activity measurement in one week-old seedlings, showing the same expression pattern as found with the GUS reporter gene. Bar = 100 μm in A-D, I and L; Bar = 50 μm in E-G.

and in situ mRNA hybridization, as opposed to a single gene-expression study as reported here. Based on the expression of the carrot EP3 genes in the integuments and in the endosperm, it was argued that the role of the corresponding proteins in somatic embryogenesis (de Jong *et al.*, 1992; de Jong *et al.*, 1993) was a reflection of their "nursing role" during zygotic embryogenesis (van Hengel *et al.*, 1998). This is difficult to sustain in the case of the *Arabidopsis AtEP3/AtchitIV* gene, that is not expressed in integuments nor in endosperm. We have only obtained indirect evidence that chitinase proteins may actually enter into the embryo sac through deposition by the pollen tube. Thus, in *Arabidopsis* the function of the AtEP3/AtchitIV class IV chitinase during embryo development may be restricted to somatic embryogenesis in tissue culture. It is possible that another member of the *Arabidopsis* class IV chitinase gene family is expressed during seed development.

The expression pattern of the *AtEP3/AtchitIV* gene during normal plant development appears highly complex and quite difficult to interpret in terms of functional significance. High levels of *AtEP3/AtchitIV* promoter activity were observed in mature pollen prior to anthesis and later in growing pollen tubes, suggesting a possible role in the male gametophyte. Being a secreted enzyme, the possible plant substrate for the AtEP3 chitinase might be found in the environment of the mature pollen grain, the locule, or in the stigma or transmitting tract of the style.

The carrot EP3 chitinase is able to cleave AGPs in vitro (van Hengel *et al.*, 2001), it co-localizes with AGPs in developing seeds, and after incubation with the EP3 chitinase, the promoting effect of AGPs on somatic embryogenesis (Kreuger and van Holst, 1993) is enhanced (van Hengel *et al.*, 2001). These results suggested that AGPs could be a substrate for the carrot EP3 chitinase. Interestingly, AGPs have also been identified in pollen and in the

transmitting tract of several plant species (e.g. Cheung *et al.*, 1995; Du *et al.*, 1996; Du *et al.*, 1994; Gerster *et al.*, 1996; Lind *et al.*, 1994) and it was proposed that they could promote pollen germination, pollen tube growth and serve as chemoattractants for their guidance (Wu *et al.*, 1995). It is therefore tempting to speculate that such AGPs can be targets for chitinases secreted by pollen.

After germination, the *AtEP3/AtchitIV* gene appears to be first expressed in root epidermal cells undergoing root hair differentiation, suggesting a correspondence between *AtEP3/AtchitIV* gene expression and tip-growing cells. Mutants such as *tip1*, impaired both in pollen tube growth and root hair elongation suggest that both processes share a common pathway (Ryan *et al.*, 1998; Schiefelbein *et al.*, 1993). Certain AGPs are also found on the root surface (Samaj *et al.*, 1999), where they may be involved in root epidermis cell elongation (Ding and Zhu, 1997).

In addition to developing root hairs and growing pollen tubes, the *AtEP3/AtchitIV* gene is expressed in stipules and hydathodes. ATHCHIA, another *Arabidopsis* chitinase, supposedly involved in plant defense (Samac and Shah, 1991), was found to be expressed in the very same organs. Nevertheless, AtEP3/AtchitIV expression did not seem to change when grown in sterile or challenging conditions, raising questions on a possible defense function, also suggested by de A. Gerhardt *et al.* (1997). Besides, many genes with unrelated functions, such as a specific marker used to study the formation of the serrated margin of leaf blades in *Arabidopsis* (Tsukaya and Uchimiya, 1997), *ENOD40* in *Sesbania rostrata* (Corich *et al.*, 1998) and *fruitful* in *Arabidopsis* (Gu *et al.*, 1998) are expressed in stipules and/or in hydathodes. One possibility exists in that expression of the *AtEP3/AtchitIV* gene in stipules and hydathodes corresponds with or just precedes cell death. Stipules are regarded by some (Medford *et al.*, 1994; Meicenheimer *et al.*, 1983) as aborted leaf primordia, while hydathodes could share the same death fate as termination of the leaf vascular system (Hugouvieux *et al.*, 1998). In addition entire early aborted zygotic embryos express this gene, suggesting a correlation with cellular status rather than with specific cell types.

Finally there is growing evidence that AGPs are involved in cell death. A study of Gao and Showalter (1999) showed that perturbation of AGPs by Yariv reagent induces programmed cell death (PCD) in *Arabidopsis* suspension-cultured cells and they propose that AGPs might be involved in other plant PCD responses as well (Dolan *et al.*, 1995; Gao and Showalter, 1999; Schindler *et al.*, 1995). Pollination in tobacco is also associated with cell death involving AGPs, showing that pollination induces deterioration of the transmitting tissue, undergoing PCD and, thus allowing easier penetration of the pollen tubes (Wang *et al.*,

1996). This deterioration is accompanied by the release of numerous factors such as chemoattractants and "growth factors" like the AGP TTS (Cheung *et al.*, 1995).

In conclusion, we propose that the AtEP3/AtchitIV chitinase we have cloned is involved in regulating PCD in cells that express the *AtEP3/AtchitIV* gene. AGPs are likely candidates for mediators in this process, some of which may require chitinase activation.

Materials and methods

Plant material

Wild-type *Arabidopsis thaliana* (L.) Heynh, plants were grown under long day light conditions from seeds germinated on wetted filter paper. The Recombinant Inbred (RI) lines used for the RFLP mapping were generated by (Lister and Dean, 1993) and obtained as seed stocks from the Nottingham *Arabidopsis* Stock Centre and were grown together with the parental ecotypes, Landsberg *erecta* (Ler) and Columbia (Col). The ecotype Ler was also the plant material used for RNA isolation and in-situ hybridization. The ecotype Wassilewskija (WS) was used for plant transformation. Transformed plants were grown in the same day light conditions, after germination on selective medium (0.5x MS salts (Duchefa, Haarlem, The Netherlands); Murashige and Skoog, 1962), 1% sucrose, 0.8% (w/v) agar and 100 μg ml^{-1} kanamycin sulphate (Duchefa).

In-vitro culture

Arabidopsis embryogenic and non-embryogenic lines were initiated and maintained as described by Mordhorst *et al.* (1998), either from seedlings with the *primordia timing* mutant, or from immature zygotic embryos for the wild-type (Ler or WS) and the *AtEP3/AtchitIV::GUS* lines. Yellowish non-embryogenic clusters were selected out of the embryogenic cultures in order to establish control non-embryogenic lines derived from the same starting material. In-vitro grown *AtEP3/AtchitIV::GUS* plants were germinated and maintained on the selective medium described above.

Identification and cloning of the AtEP3/AtchitIV gene

The *AtEP3/AtchitIV* cDNA, *tai224* (GenBank accession number Z26409) was identified in the dbEST database with the *Daucus EP3* gene as query sequence. The *tai224* clone was obtained from the Institut de Biologie Moléculaire des Plantes of Strasbourg (France) as a full length, partially sequenced (375 bases) cDNA, inserted in pBluescript® SK^-

(Stratagene, La Jolla, Calif., USA). Complete sequencing was then performed at our laboratory. The full-length clone (822 bp) was used inserted in its original vector (ptai224).

The genomic library screened, kindly provided by Carlos Alonso-Blanco (Wageningen University, The Netherlands), was constructed in the Lambda Fix®II vector (Stratagene) from the ecotype Ler. The library was screened with the [^{32}P]-labeled tai224 full-length cDNA and 5 clones were isolated and subcloned into pBluescript® SK$^-$ (Stratagene). Restriction mapping and sequencing showed that all clones were identical and truncated at their 3'end. Full-length genomic clones were subsequently isolated by PCR on Ler and Col genomic DNA, using primers designed against both ends of the cDNA (TAI1: 5'-AAAATGTTGACTCCCACCATTTCTAAATCC-3' and TAI2: 5'-TGTTAGCAAGTGAGGTTGTTTCCAGGATCA-3'). Sequence analysis of the different clones revealed no difference between these 2 ecotypes. It also revealed that the *tai224* genomic sequence was identical to the one of *AtchitIV* submitted to the EMBL database by de A. Gerhardt *et al.* (1997) (Accession number Y14590).

Protein purification, and Western blotting

Total protein extracts from *Arabidopsis* and carrot embryogenic and non-embryogenic cell suspension culture media were purified as described by (van Hengel *et al.*, 1998). Total proteins from *Arabidopsis* and carrot were separated by SDS-PAGE according to (Laemmli, 1970) and subsequently transferred to an Immobilon™-P PVDF Transfer Membrane (Millipore, Bedford, MA, USA). Immunological detection was performed, as described by de Jong *et al.* (1995) with rabbit antiserum raised against EP3.

Mapping of AtEP3/AtchitIV

Mapping by RFLP was performed as described by Lister and Dean (1993). Genomic DNA was extracted from each line according to Reiter *et al.* (1992) and 5 µg parental genomic DNA (Ler and Col) was restricted with 25 U of each of six different enzymes (*Bam*HI, *Bgl*II, *Cla*I, *Dra*I, *Eco*RI and *Hind*III) in 1x KGB buffer (100 mM potassium glutamate, 25 mM Tris-acetate (pH 7.5), 10 mM MgAc, 50 µg ml^{-1} BSA (fraction V, Sigma), 0.5 mM ß-mercaptoethanol) containing 1 mM spermidin, separated on a 1% (w/v) agarose gel, and transferred to a Nytran-Plus membrane (Schleicher & Schuell, Dassel, Germany) following the manufacturer's recommendations. Hybridization with the complete *tai224* cDNA, [^{32}P]-labeled using Random Primer Labeling, was carried out overnight at 65°C in

1.5% (w/v) SDS, 10% (w/v) dextran sulphate and 100 μg ml^{-1} denatured salmon sperm DNA. Filter was washed at 65°C in 5x SSC (1x SSC: 150 mM NaCl, 12 mM sodium-citrate, pH7.0), 0.1% (w/v) SDS and 2x SSC, 0.1% (w/v) SDS (15 min each) before exposure to an X-ray film at -80°C for 2 d. Segregation of the revealed polymorphism over the RI line population was analyzed according to the same protocol, using 400 ng of genomic DNA from each line restricted with the polymorphic enzyme. The data were scored as L (Ler), C (Col) and U (unclassified) and sent to the Nottingham *Arabidopsis* Stock Centre for linkage analysis and mapping of the marker towards the markers mapped previously.

Physical mapping was performed by hybridization of the [^{32}P]-labeled *tai224* cDNA to the CIC YAC library (Creusot *et al.*, 1995) kindly provided to us by Jo West (John Innes Centre, Norwich, UK). Membranes were prehybridized for 2 h at 62°C in hybridization buffer (0.25 M NaCl, 0.25 M Na_2HPO_4, 10% (w/v) PEG 6000, 7% (w/v) SDS, 1mM EDTA). The probe was added and hybridization performed for 24h at 62°C. Following hybridization the membranes were rinsed at room temperature in 3xSSC, 0.1% (w/v) SDS and then washed at 62°C for 20 min in the same solution, followed by 20 min in 0.1xSSC, 0.1% (w/v) SDS and subsequently exposed to an X-ray film. The map location was obtained from David Bouchez (INRA Versailles, France), according to the coordinates of the hybridizing YACs.

Fluorescence in situ hybridization and subsequent immunocytochemical detection were carried out as described by Fransz *et al.* (1998). A 15Kb lambda clone containing the genomic sequence of *AtEP3/AtChitIV* was used as a probe.

Analysis by RT-PCR

RT-PCR was carried out as described by van Hengel *et al.* (1998) using 9514PP (5'-AAAATGTTGACTCCCACC-3') as upstream primer and 9515PP (5'-TGTTAGCAAGTGAGGTTG-3') as downstream primer for amplification of the *AtEP3/AtchitIV* reversed transcribed cDNA. Ubiquitin was amplified as a control of the RNA using 5'-TAGAAGCTTATGCAGAT$^{C}/_{T}$TTTGTGAAGAC-3'and 5'TATGGATCCACCA CCACG$^{G}/_{A}$AGACGGAG-3' as upstream and downstream primers respectively (Horvath *et al.*, 1993). PCR products were analyzed by agarose gel electrophoresis and Southern blot analysis was performed, as described above, on the AtEP3/AtchitIV samples using the full-length *tai224* cDNA as a probe.

Construction of AtEP3/AtchitIV promoter::reporter fusions, plant transformation and analysis of the reporter gene expression

The *AtEP3/AtchitIV* promoter::reporter constructs were made in a 2-step cloning. The *AtEP3/AtchitIV* promoter was first cloned into pBluescript® SK⁻ (Stratagene) as a *Pst*I-*Xba*I 1100bp fragment ending directly upstream the *AtEP3/AtchitIV* ATG, after introduction, by PCR mutagenesis, of a *Xba*I site at the 3'end, using the primers 5'pTAI (5'-CCCTGCAGATCTTCCTGG-3') and 3'pTAI (5'-GCTCTAGATTTGATGTTGTTGAGG-3'). From pBluescript® the promoter was cloned in the GUS binary vector pGPTV-KAN (Becker *et al.*, 1992) as a *Sal*I-*Xba*I fragment and as a *Sst*I-*Kpn*I fragment in the luciferase binary vector pMT500 (Toonen *et al.*, 1997). The constructs were transformed into *Arabidopsis* plants, ecotype WS, by vacuum infiltration according to Bechtold *et al.* (1993). Transformants were selected on kanamycin at each generation and homozygous T_3 plants were assayed for reporter gene expression together with control plants transformed with the same binary vector, but having the GUS or luciferase gene driven by the CaMV 35S promoter. Seeds of these plants were kindly provided by Valérie Hecht (Wageningen University, The Netherlands). Histochemical GUS assays were performed as described in Vroemen *et al.* (1996), using 2mM of potassium ferri- and ferrocyanide and staining at 37°C for up to 5 d. Luciferase activity was measured as described in Toonen *et al.* (1997).

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Chapter 3

Expression of gametophytic markers during *Arabidopsis thaliana* somatic embryogenesis

Abstract. Plant cells have the remarkable ability to be totipotent and can be triggered to form somatic embryos under the proper conditions. This provides a convenient system to study the mechanism(s) by which somatic cells acquire their embryogenic potential and undergo the specific patterning required for the formation of an embryo. Very little is known about the genes involved in these processes and even less about the analogies between zygotic and somatic embryogenesis in terms of genetic control. We have addressed this question in *Arabidopsis thaliana* by studying the expression pattern of gametophytic marker genes during somatic embryogenesis. The analysis of their respective expression patterns revealed that all markers tested are also expressed during somatic embryogenesis. Their expression patterns appeared to be restricted both spatially and temporally, suggesting that they had retained a certain degree of specificity and that at least a number of gametophytic gene expression programs are conserved in somatic embryogenesis.

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Introduction

Somatic embryogenesis has been frequently employed to identify genes preferentially expressed during early plant embryogenesis. This is based on the premise that there is substantial similarity between genetic programs controlling zygotic and somatic embryo development (for reviews see Yeung (1995) and Mordhorst *et al.*, 1997). Far less is known about genes that are involved in the acquisition of embryogenic competence. In *Arabidopsis thaliana*, recessive mutations in different genes result in the formation of somatic embryos under in vitro conditions (Mordhorst *et al.*, 1998; Ogas *et al.*, 1997), while ectopic expression of LEC1 was reported to give spontaneous somatic embryo formation (Lotan *et al.*, 1998). Other genes such as the carrot SERK gene were identified based on their expression in embryogenic cells (Schmidt *et al.*, 1997).

It is not clear which phase of zygotic embryogenesis is equivalent to embryogenic cell formation in vitro. Cells that show signs of chromosome reduction and morphological features of gametophytic cells such as immature mononucleate pollen or embryo sac cells, were identified in carrot embryogenic cultures (Giorgetti *et al.*, 1995). This suggests that during somatic embryogenesis some cells pass through a gamete-like state that may be a prerequisite for embryogenic cell formation. The in planta expression pattern of genes such as *AtSERK1* (Hecht *et al.*, submitted) and *AtEP3* (Passarinho *et al.*, 2001) supports this hypothesis. The *AtSERK1* gene is first expressed in ovule primordia, subsequently in the female gametophyte and finally in the zygotic embryo (Hecht *et al.*, submitted). The *AtEP3* gene is expressed in several somatic cell types but predominantly in mature and germinating pollen (Passarinho *et al.*, 2001). These observations show that cells present in embryogenic cultures may share gene expression programs with gametophytic cells.

In this work we reversed this approach and asked whether the expression of specific gametophytic marker genes was also detectable during *Arabidopsis thaliana* somatic embryogenesis. Markers for all elements of the embryo sac have been identified while screening an enhancer-trap collection for such genes (Grossniklaus, unpublished data). We have made use of a number of these markers to trace the expression of ovule genes during somatic embryogenesis in *Arabidopsis*. As marker for the male gametophyte, we have used the LAT52 promoter fused to β-glucuronidase (GUS; Eady *et al.*, 1994). Interestingly, all markers tested appear to be expressed during *Arabidopsis* somatic embryogenesis in highly specific and restricted patterns, suggesting a correspondence between genes expressed in embryogenic cells in vitro and in gametophytic cells in planta.

Results

Expression pattern of gametophytic markers in vitro

Embryogenic cultures were initiated from all *pt* lines harboring the different ET constructs and from the LAT52::GUS marker line. The expression pattern of these markers in planta is summarized in Table 1. Three weeks after initiation, embryogenic and non-embryogenic cultures of each line were maintained separately and subcultured weekly. Expression of the different markers was followed over a period of two months by GUS histochemical localization. Stainings were performed weekly upon subculturing of each marker line. The results obtained are summarized in Table 2. All markers tested this way were expressed in a temporally and spatially different fashion.

Marker line	GUS expression pattern in ovules
ET127	Synergids
ET133	Stripe at chalazal pole and megagametophyte
ET184	Dorsal micropyle or synergids
ET204	Synergids and funiculus
ET1081	Egg cell and synergids
LAT52::GUS	Microspores from late uninucleate stage onwards

Table 1. Marker lines used for the female and male gametophyte
The expression patterns of the ET lines were observed while screening for genes involved in ovule and early seed development (Grossniklaus, unpublished results). GUS stainings were performed as described in (Vielle-Calzada *et al.*, 2000). The expression pattern of LAT52::GUS is fully described in (Eady *et al.*, 1994).

Lines ET127 and ET204 both mark the synergids in planta and they have expression patterns quite similar to one another in vitro as well, as can be seen by comparing Figure 1A-D (ET127) with Figure 1E-H (ET204). The only difference is that the expression level of ET204 is lower than that of ET127. GUS staining is detected in cells of non-embryogenic clusters (nec) present in embryogenic cultures (Figure 1A, 1B and 1E-G). These clusters are yellowish to whitish and have a "rougher" surface than embryogenic clusters (ec) that are bright green and smooth. When subcultured, non-embryogenic clusters only proliferate and no embryos arise from such calli, whereas embryogenic clusters continuously develop embryogenic structures that are able to form embryos when cultured in hormone free medium

(Mordhorst *et al.*, 1998). In embryogenic cultures the ET127 and ET204-expressing cells are located in those parts of the callus that are in close vicinity to the developing embryogenic structures (Figure 1A, 1B and 1E-G). No expression of either of these markers was ever seen in the embryogenic structures themselves. Upon subsequent subculturing of non-embryogenic cultures without visual selection for green embryogenic clusters any remaining embryogenic cells are rapidly lost (Mordhorst *et al.*, 1998). This is accompanied by an increased expression of both ET127 and ET204 to an almost equal level (compare Figure 1C with 1D and 1H). In embryogenic cultures expression of both markers remains constant and is fully comparable to what is shown in Figure 1A, B and 1E-G. This suggests that the expression of ET127 and ET204 is negatively correlated with the presence of embryogenic cells. In addition, their expression is not homogeneously distributed and seems to be confined to certain sectors of the non-embryogenic calli (Figure 1D and 1H). This shows that not all cells are identical in these non-embryogenic calli and some level of differentiation is apparently maintained in culture.

In line ET133 (Figure 1I-L), GUS staining is spatially less restricted in embryogenic calli (compare Figure 1B or 1F with 1J) and is even occasionally observed in the basal end of the embryogenic structures (Figure 1J and 1K). However, all three markers are expressed in comparable regions of the calli. Remarkably, this mimics the situation in planta where ET127 and ET204 expression (synergids) is much more restricted than the one of ET133 (entire embryo sac). ET133 expression is not restricted to embryogenic calli, but is also apparent in non-embryogenic calli (Figure 1L), although more abundantly in the former (Figure 1J). In contrast to ET127 and ET204, which appear to be more abundantly expressed in non-embryogenic calli, ET133 expression is weaker in these calli and decreases in time with subculturing. Yet, the expression pattern of all three markers in non-embryogenic calli is rather similar in its heterogeneity (compare Figures 1D, 1H and 1L). Unfortunately, it is very difficult to identify morphologically different cell types in these stained calli and therefore we cannot answer whether the cells marked by ET127 and ET204 are indeed the same cells that are also marked by ET133.

In planta, lines ET184 and ET1081 have slightly different expression patterns, being restricted to the micropylar region or the synergids and the egg cell or the synergids respectively (Grossniklaus, unpublished results). However, in culture their expression pattern is rather similar (see Figure 1M-O for ET184 expression and P-R for ET1081 expression). It is more restricted than the ones of ET127, ET204 and ET133. In embryogenic cultures, ET184 GUS staining was only observed in a few cells in the close vicinity of developing embryogenic clusters (Figure 1M). Somewhat later the expression becomes stronger but remains restricted,

reminiscent of the pattern observed for ET133 much more restricted both spatially and in intensity. GUS staining was only observed in some of the calli (compare Figures 1O with 1D, 1H and 1L).

Marker line	GUS expression pattern during somatic embryogenesis
ET127	High expression level (2h staining) Never expressed in green embryogenic clusters Maximum expression in non-embryogenic (NE) cultures Negatively correlated with embryogenicity, but never uniform expression in NE calli
ET204	Identical to ET127, but lower expression level (4h staining)
ET133	High expression level (2h staining) Maximum expression in embryogenic cultures, some expression in green embryogenic clusters and in starting NE cultures Positively correlated with embryogenicity
ET184 and ET1081	Low expression level (overnight staining) Restricted to a few sectors of NE callus in both embryogenic and NE cultures Never expressed in green embryogenic clusters No apparent correlation with embryogenicity
LAT52::GUS	Low expression level (overnight staining) Expression restricted to NE callus and older embryogenic clusters both underlying developing embryogenic clusters Correlated with embryogenicity? (*)

Table 2. Summary of GUS expression patterns during somatic embryogenesis.
(*) The expression of the LAT52::GUS marker could not be analyzed in non-embryogenic cultures initiated from the same starting material.

Expression of ET1081 that of all markers tested here shows the most restricted expression in planta, also showed the most restricted pattern in culture. In Figure 1P only a few stained cells are seen in torpedo stage embryos (Figure 1Q), while in non-embryogenic cultures, similar to in embryogenic ones, only a few cells show GUS staining.
Thus, all of the five markers tested show subtle differences in expression patterns. None is specific for embryogenic cultures only, and most remarkably, all seem to maintain a certain restriction in expression as also found in planta.

The reverse seems also true, since the *AtSERK1* gene that was identified as a marker for single embryogenic cells in carrot (Schmidt *et al.*, 1997) is expressed in the ovule primordia, in the entire female gametophyte and later during zygotic embryogenesis in

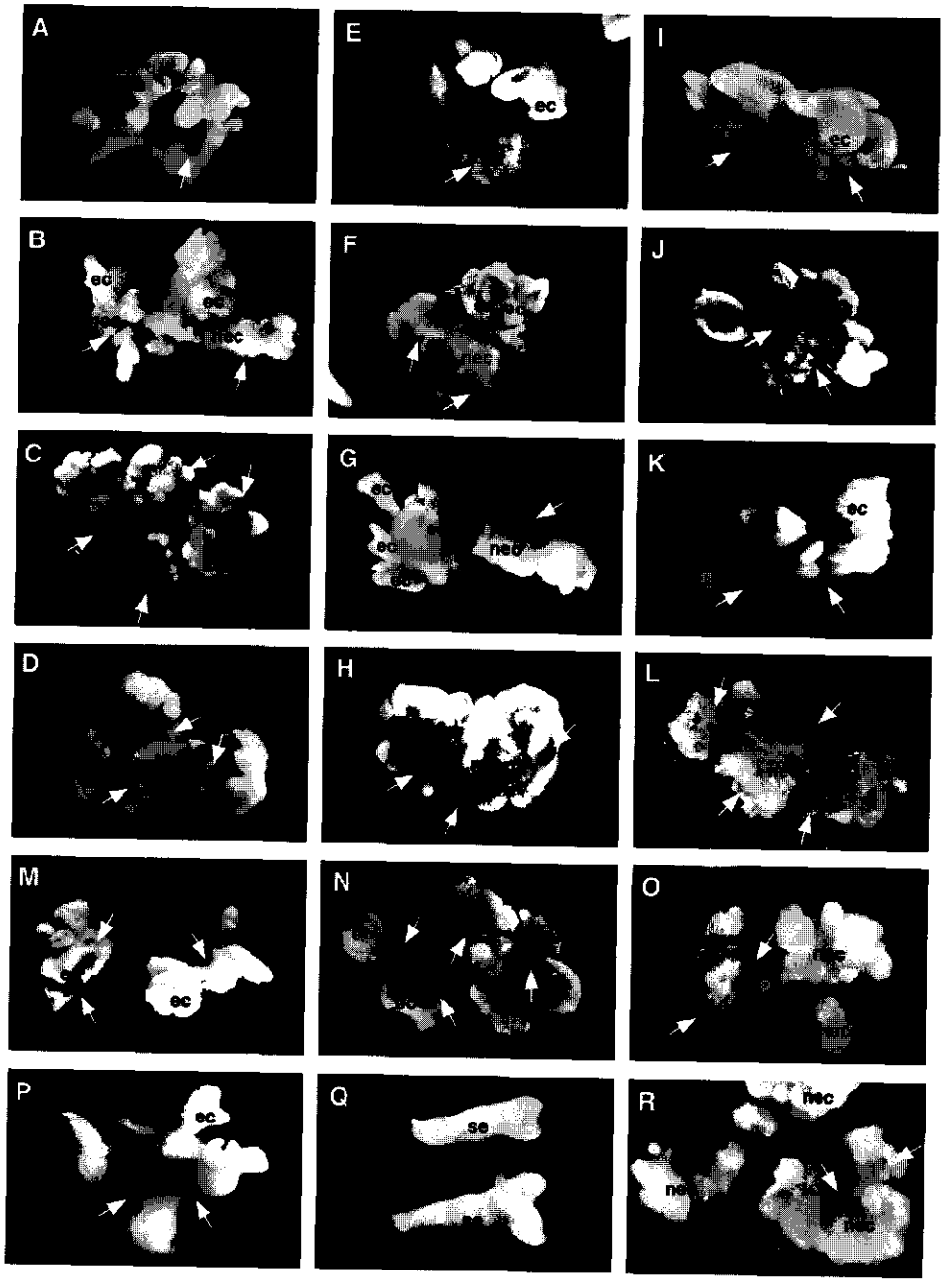
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Figure 1. GUS histochemical localization of female gametophytic markers expression during somatic embryogenesis.
A-D. Expression pattern of the synergid marker ET127. (A-B) embryogenic calli; (C-D) non-embryogenic calli with decreasing embryogenic potential. **E-H.** Expression pattern of the synergid marker ET204. (E-G) embryogenic calli; (H) non-embryogenic callus. **I-L.** Expression pattern of the megagametophyte marker ET133. (I-K) embryogenic calli; (L) non-embryogenic calli. **M-O.** Expression pattern of the synergid or dorsal micropyle marker ET184. (M-N) embryogenic calli; (O) non-embryogenic callus. **P-R.** Expression pattern of the egg cell and synergid marker ET1081. (P) embryogenic callus; (Q) embryos isolated from hormone-free medium; (R) non-embryogenic calli. Examples of positive cells are indicated by the arrows. (ec) embryogenic cluster; (nec) non-embryogenic cluster, (se) somatic embryo.

Arabidopsis (Hecht *et al.*, submitted). In embryogenic cultures the *AtSERK1* promoter is active in embryogenic clusters, as can be seen in Figure 2A (see also Hecht *et al.*, submitted). GUS staining is restricted to some of the developing embryogenic structures probably reflecting specific stages of their development among those present in this embryogenic cluster. Another gene we identified for its role during carrot somatic embryogenesis (de Jong *et al.*, 1992) was also studied in *Arabidopsis* (Passarinho *et al.*, 2001). The *AtEP3* endochitinase gene is specific for embryogenic cultures where its promoter is active in embryogenic clusters but not in developing embryogenic structures themselves (Figure 2B and Passarinho *et al.*, 2001). Interestingly, in planta the gene is not expressed in the female gametophyte, but in mature and germinating pollen instead. In addition, the *AtEP3* gene is also expressed in somatic cells in planta (root hairs, hydathodes and stipules; Passarinho *et al.*, 2001).

To answer if other genes more specifically expressed in the male gametophyte are also expressed during somatic embryogenesis, we analyzed the expression of a specific pollen marker gene during somatic embryogenesis. In *Arabidopsis*, the LAT52 promoter is activated in late uninucleate microspores immediately prior to microspore mitosis (Eady *et al.*, 1994) and remains active throughout the early stages of pollen tube growth (Twell *et al.*, 1998).

We found that during somatic embryogenesis the LAT52 promoter is also activated and appears to be regulated both spatially and temporally. Green embryogenic clusters arise from the shoot meristem area of the germinating seedling and the first signs of LAT52 promoter activity are observed in this region (Figure 3A and B). As these clusters proliferate, GUS staining remains localized in the cells underlying them and is absent from the embryogenic structures themselves (Figure 3C and D).

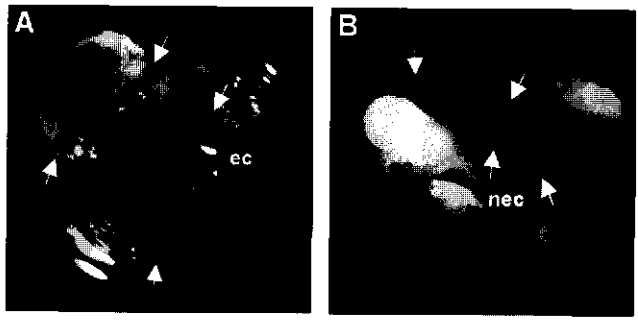

Figure 2. GUS histochemical localization of *AtSERK1::GUS* and *AtEP3::GUS* expression during somatic embryogenesis.
A. Expression of *AtSERK1::GUS* in an embryogenic callus. Some of the positive embryogenic structures are indicated by the arrowheads. **B.** Expression of *AtEP3::GUS* in an embryogenic callus. Some of the positive cells in the non-embryogenic cells are indicated by the arrowheads. (ec) embryogenic cluster; (nec) non-embryogenic cluster.

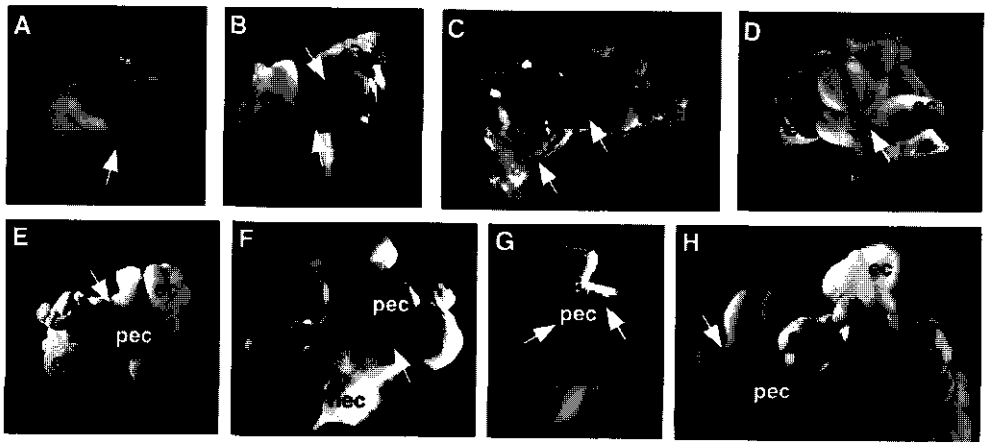

Figure 3. GUS histochemical localization of *LAT52::GUS* expression during somatic embryogenesis.
A. 3-week old embryogenic callus, with developing embryogenic clusters arising from the shoot meristem region. **B.** 4-week old embryogenic callus. The shape of the germinating seedling from which it arose is still visible. The cotyledons were overgrown by developing embryogenic clusters. **C-E.** More developed embryogenic clusters. **F-G.** Embryogenic calli, where development of secondary embryogenic clusters is visible on top of older ones. **H.** Higher magnification of an embryogenic cluster. Positive cells are indicated by the arrows. (ec) embryogenic cluster, (nec) non-embryogenic cluster; (pec) primary embryogenic cluster on top of which new embryogenic clusters (ec) form.

At later stages, secondary embryogenic clusters form continuously on top of each other (Mordhorst *et al.*, 1998). LAT52 expression seems to follow this cycle since the promoter

activity spreads to the first embryogenic clusters that were up to that point devoid of activity (Figure 3E-H). However, we could not verify whether the activity of the LAT52 promoter was directly linked to embryo formation since we were not able to establish any LAT52::GUS non-embryogenic lines from the same material.

Discussion

In the present work we have shown that markers expressed in the female and in the male gametophyte are also expressed during somatic embryogenesis. The expression pattern of these markers was not uniformly distributed in cultured cells and appeared to be restricted both spatially and temporally. From this observation, a number of general conclusions can be drawn.

Markers such as ET127 and ET204 that are expressed in the same cell type in the plant (in this case the synergids) are also expressed in an almost identical pattern in tissue culture. This parallel could also be observed in the case of the embryo sac marker ET133 and the ET1081 and ET184 markers. This is quite remarkable, in view of the completely different level of tissue organization in planta and in tissue culture. However, when comparing markers in tissue culture with one another, differences were also observed. For instance, the expression pattern of the synergid marker ET184 should be more similar to the one of ET127 or ET204 and yet appears quite different. Apparently markers that are expressed in the same cell type in the embryo sac can be part of different cellular mechanisms. This may become visible in terms of expression pattern when the cells are placed in the different context of a culturing system.

A second general conclusion is that except for ET133 none of the female gametophytic markers are expressed in somatic embryos themselves. This mimics the situation as it is in planta and it is not an artifact of our system because zygotic embryo-expressed genes such as AtSERK1 (Hecht *et al.*, submitted) are also expressed in embryogenic clusters as well as in embryos.

A third general conclusion comes from looking at the correlation with embryogenicity. Expression of the synergid markers ET127 and ET204 does indeed increase as cultures lose their embryogenic potential. In plants synergids degenerate after fertilization and upon development of the embryo. One could assume that they would remain expressed in case no embryo was formed and still express a number of genes among which these markers. Non-embryogenic cultures could mimic such a situation and explain our observations. In contrast,

other markers like ET133 appear to be positively correlated with embryogenic capacity. ET133 marks all cells of the embryo sac in planta, i.e. including the egg cell and the central cell. One could imagine that some of the gene expression programs in these cells would remain active during early embryogenesis. This is apparently what happens in these embryogenic cultures where ET133 is the only marker that is expressed to a low level in the basal end of the developing embryogenic cluster. On the other hand, in non-embryogenic cultures these functions would no longer be needed and only some of the ET133 expression seems to remain, which could in fact be due to the partial "synergid" expression pattern of this marker.

The same assumptions can be made for LAT52::GUS, although we could not analyze its expression in non-embryogenic cultures and compare it with other specific male gametophytic markers. The LAT52 promoter activity is precisely regulated, closely accompanying embryo development. The cells where it is active are always underneath newly developing embryogenic clusters resembling the expression pattern of the *AtEP3* chitinase gene that is also expressed in mature pollen (Passarinho *et al.*, 2001). These last results are rather intriguing, suggesting that not only maternal tissues need to be present for embryos to develop in vitro (van Hengel *et al.*, 1998) but that a paternal contribution might be required as well. This also supports the findings of Giorgetti *et al.* (1995) who observed cells with the morphological features of immature mononucleate pollen in carrot embryogenic cultures.

The expression patterns of these genes and markers also confirm previous observations made in carrot embryogenic suspension cultures in which it was shown that various cell types co-exist and have different fates and functions (McCabe *et al.*, 1997; Toonen *et al.*, 1994). For instance, the immunolocalization of the specific arabinogalactan epitope JIM8 (Pennell *et al.*, 1991) in carrot embryogenic cultures revealed that a certain type of cells first has and then becomes devoid of the JIM8 epitope. Further work suggested that it was the JIM8-negative cells that develop from these JIM8-positive cells that are competent to form embryos in culture (McCabe *et al.*, 1997) The JIM8-positive cells present in these cultures would then play the nursing role of the seed endosperm allowing the formation and the development of the embryo, although the exact mechanisms of this communication in vitro might differ from those occurring in a developing seed. The localization of the JIM8 epitope in the cell wall of gametophytic cells in planta - e.g. *Brassica* sperm and *Lilium* sperm and generative cells (Southworth and Kwiatkowski, 1996); micropyle of *Amaranthus hypochondriacus* ovules (Coimbra and Salema, 1997) – is also well established following

earlier observations of its occurrence in *Brassica* young embryos but also prior to that in the nucellar epidermis, synergid cells, and the egg cell (Pennell *et al.*, 1991).

In conclusion, the results we describe in this work support the hypothesis that a number of pathways are shared between gametogenesis and embryogenic cell formation in vitro.

Materials and methods

Plant material

Enhancer-trap (ET) lines marking female gametophytic cells (Table 1 and Vielle-Calzada *et al.*, 2000) were crossed into the *primordia timing* (*pt*) mutant background in which somatic embryogenesis is highly facilitated (Mordhorst *et al.*, 1998). Subsequent generations of this cross were selected on kanamycin for co-segregation of the *pt* mutant phenotype and the enhancer-trap construct. F_3 seeds, homozygous for both traits, were used for analysis. These plants, and LAT52::GUS homozygous plants (Eady *et al.*, 1994) were grown under long day light conditions after germination on selective medium containing 0.5x MS salts (Duchefa, Haarlem, The Netherlands; Murashige and Skoog, 1962), 1% (w/v) sucrose, 0.8% (w/v) agar and 100 $\mu g\,ml^{-1}$ kanamycin sulphate (Duchefa).

Initiation of embryogenic marker lines

Embryogenic lines were initiated as described by Mordhorst *et al.* (1998). Homozygous F_3 seeds from crosses between *pt* and the different ET marker lines were directly germinated in liquid medium containing 2,4-dichlorophenoxyacetic acid (2,4-D; Mordhorst *et al.*, 1998). *LAT52::GUS* embryogenic lines were initiated without prior crossing with the *pt* mutant, using dissected immature zygotic embryos from *LAT52::GUS* homozygous plants.

Cultures were selected after 3 weeks for the presence of embryogenic and non-embryogenic calli. From that point on, embryogenic and non-embryogenic cultures were maintained separately and subcultured on a weekly basis.

GUS histochemical localization

GUS expression was determined by histochemical staining of both embryogenic and non-embryogenic tissues as described in Passarinho *et al.* (2001). Stainings were repeated

weekly for a period of two months for relevance of the results that might be affected by the stage of the different cultures. Observations were done with a Nikon SMZ-2T binocular.

Acknowledgements

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Chapter 4

A search for *AtEP3* null mutants by insertional mutagenesis: screening and phenotypic analysis

Abstract. The EP3 class IV endochitinase (EC 3.2.1.14) was shown to play a crucial role in somatic embryo development in carrot (*Daucus carota* L.). We have previously identified the *Arabidopsis thaliana* (L.) Heynh ortholog AtEP3 and analyzed its expression pattern during development. In this work, we have searched for insertions into the *AtEP3* gene and for this purpose we have screened two plant collections representing over 80,000 T-DNA and transposon insertions. A total of six insertions were identified and the corresponding plants were analyzed for putative mutant phenotypes. We report here the details of the screening and of the phenotypic analysis, which showed that the absence of AtEP3 protein does not result in any visible developmental phenotype under regular growth conditions. To our knowledge, this is the first report of a reverse genetics approach in an attempt to understand the function of one of the 24 *Arabidopsis* chitinase genes.

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Introduction

Transposon and T-DNA insertional mutagenesis has been widely employed in *Arabidopsis* and other plant species to tag genes and generate knockout mutants (Aspiroz-Leehan and Feldmann, 1997; Speulman *et al.*, 1999). Pooling collections of insertional mutants has made it feasible to screen for insertions in any given gene of interest by a PCR-based approach. This has been successfully used in *Arabidopsis* (Bouchez and Hofte, 1998; Geelen *et al.*, 2000; Papi *et al.*, 2000) and provides a powerful method for functional gene analysis, allowing the study of gene families (Meissner *et al.*, 1999; Winkler *et al.*, 1998).
We have searched for insertional mutants in the *Arabidopsis AtEP3* endochitinase gene (Passarinho *et al.*, 2001). The *AtEP3* gene was identified as the *Arabidopsis* ortholog of the carrot *EP3* endochitinase gene. The carrot EP3 protein was shown to play a crucial role during carrot somatic embryo development (de Jong *et al.*, 1992). In *Arabidopsis*, the *AtEP3* gene was genetically and physically mapped but no mutation was associated with the AtEP3 locus. The *AtEP3* gene is expressed in a complex pattern, most conspicuously in the male gametophyte, stipules and hydathodes, as well as in the root epidermis and emerging root hairs. In cultured cells, the gene is only expressed in embryogenic cultures (Passarinho *et al.*, 2001). No firm conclusion could be made with respect to its function in planta, except that the AtEP3 chitinase may have multiple roles during development. Generating AtEP3 null mutants was therefore essential to verify this hypothesis and understand the role of the AtEP3.

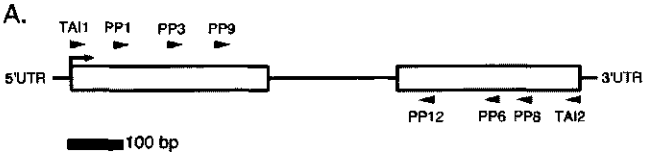
For this purpose, we have screened by PCR two different populations of plants containing T-DNA (the Versailles lines) or transposon insertions (the AMAZE lines), representing in total more than 80,000 independent insertion sites. The screening of both collections was successful and allowed the identification of one T-DNA insertion and five *En-1* insertions in the *AtEP3* gene. We report here that one of the *En-1* insertions resulted in the complete absence of AtEP3 chitinase into the medium of embryogenic cultures. Neither in culture nor in plants, any consistent developmental mutant phenotype was associated with the disruption of the *AtEP3* gene. In this work, we describe the details of the screening procedure as well as the phenotypic analysis.

Results

Screening of the Versailles T-DNA collection

AtEP3 gene-specific primers were designed according to INRA's recommendations (Figure 1A). Sixteen primer pairs were tested under simulated screening conditions on total genomic DNA from the ecotype WS (the genetic background of the T-DNA lines) using amounts of DNA between 100 and 0.1 ng. Eventually, the primers PP1 and PP6 gave the best amplification results and were used for the screening in combination with two T-DNA primers (TAG3 and TAG6, Figure 1B) to cover the possible orientations of the inserted T-DNA.

A.

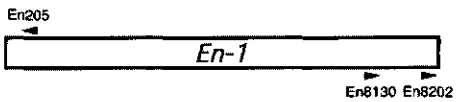


TAI1	5'-AAAATGTTGACTCCCACCATTTCTAAATCC-3'
TAI2	5'-TGTTAGCAAGTGAGGTTGTTTCCAGGATCA-3'
PP1	5'-TTCGTCAGAGCTATGTTGTAGTCAGTTTGG-3'
PP3	5'-CTCCTTCCCCTGCAAATGGTGTCTCTGTGG-3'
PP6	5'-CCACAAGGCGGTTTTGAAGGATATGACTGG-3
PP8	5'-GTTAATGGCACGGATTGTTGCACCAAAACC-3'
PP9	5'-TTGAGGCCTTAGACTCATATTCTCGTTTCG-3'
PP12	5'-TATTGTGTTGCATTCTCGTCGCAGTAATCC-3'

B.

TAG1	5'-GGACGTAACATAAGGGACTGAC-3'
TAG3	5'-CTGATACCAGACGTTGCCCGCATAA-3'
TAG5	5'-CTACAAATTGCCTTTTCTTATCGAC-3'
TAG6	5'-CACTCAGTCTTTCATCTACGGCA-3'

C.



En205	5'-AGAAGCACGACGGCTGTAGAATAGGA-3'
En8130	5'-GAGCGTCGGTCCCCACACTTCTATAC-3'
En8202	5'-CACTCCTTACCTTTTTTCTTGTAGTG-3'

***Figure 1.* Primers used for screening the Versailles and AMAZE collections.**
A. Positions (arrowheads) and sequences of the *AtEP3/AtchitIV* primers on its genomic sequence. The open boxes represent the two exons of the gene and the arrow the start codon. **B.** Positions and sequences of the T-DNA-specific primers. **C.** Positions and sequences of the *En-1*-specific primers.

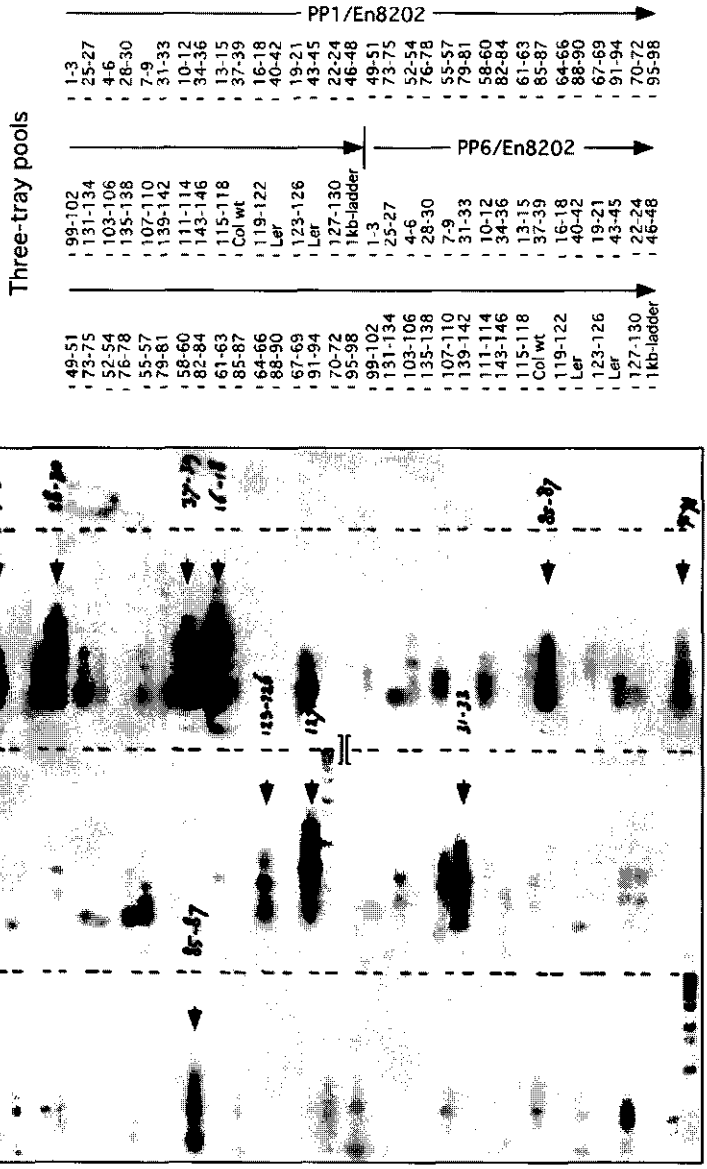


Figure 4. First round of the screening of the AMAZE collection. Identification of the three-tray pools with an *En-1* insertion in the *AtEP3* gene.
Autoradiogram of the PCR performed on the three-tray pools using primer combinations PP1/En8202 and PP6/En8202. The loading pattern of the gel is indicated above the autoradiogram. The numbers represent the tray pools contained in each three-tray pool (e.g. 1-3 contains the tray pools 1, 2 and 3). The negative controls for the PCR reaction were performed on wild-type DNA from the ecotypes *Columbia* and *Landsberg erecta* and were loaded in the lanes marked Col wt and Ler respectively. The arrowheads show the positive three-tray pools.

primers were then tested individually for aspecific amplification with each *En*-specific primer (Figure 1C).

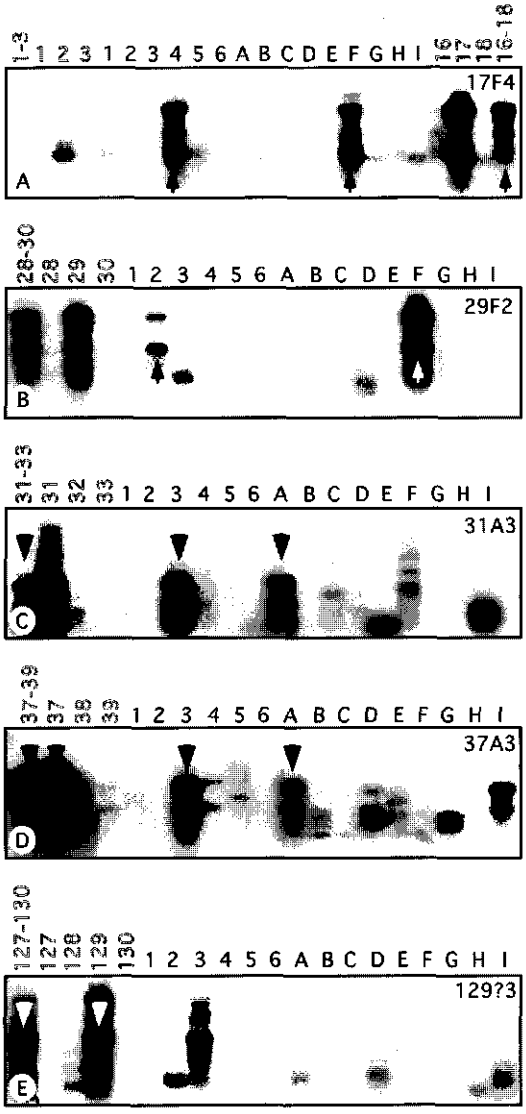


Figure 5. Second round of the screening of the AMAZE collection. Confirmation of the positive three-tray pools and identification of the corresponding individual lines.
Autoradiograms of the PCRs performed on all pooling dimensions contained in the identified three-tray pools, after hybridization with an *AtEP3*-specific probe. The PCR reactions were performed either with PP1/En8202 (A, B, D and E) or with PP6/En8202 (C). The outlined numbers represent the three-tray and single tray pools, the plain numbers the rows and the capital letters the columns. The arrowheads indicate the bands specifically amplified in all 4 dimensions (three-tray, single tray, column and row) allowing the identification of individual lines, e.g. 17F4 in A: three-tray 16-18, tray 17, column F and row 4.

No amplification product was observed. The screening was subsequently performed on the three-tray pools, and it appeared that on this material the En8130 primer produced many unspecific amplifications with both gene-specific primers (data not shown). It was therefore decided to use the En8202 primer for the *En*-3'end, which considerably improved the quality

of the PCR amplifications and allowed the identification, after hybridization, of nine positive three-tray pools (Figure 4). The same PCR reactions were repeated on each of the positive three-tray pools and the three other dimensions for these pools. Positives were confirmed when the same PCR product was amplified in all dimensions and that the coordinates obtained corresponded to an individual line (Figure 5). This brought the number of true positives down to four, with the tray-column-row coordinates 17F4, 29F2, 31A3 and 37A3 (Figure 5A-D; these coordinates were subsequently used as identification number for the insertion lines) plus an additional positive for which the column dimension was unclear (Figure 5E). All positive coordinates were confirmed by nested PCR using PP3/En8202 and PP12/En8202, for the lines identified with PP1 and PP6 respectively and by amplifying the other end of the insertion sites, using the En205 primer with TAI1 or TAI2. For the positive 129?3, without column coordinate, the reaction was repeated on each column pool, which allowed the identification of column C, giving the coordinates 129C3 (data not shown). Amplification products were purified, cloned and sequenced.

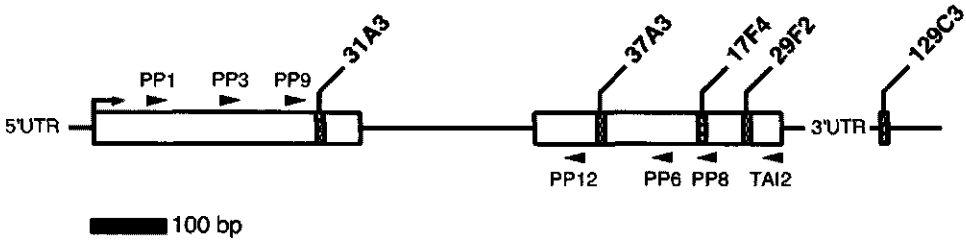


Figure 6. Position of the *En-1* insertions in the *AtEP3* gene.
En-1 insertions are depicted by the shaded boxes accompanied by the number of the line in which the element is present at this position. The exact insertion sites were determined by sequencing of the PCR products generated with the *AtEP3* primers (arrowheads) in combination with the En8202 and En205 primers.

Sequence analysis revealed, as expected, that all lines identified had an insertion into the *AtEP3* gene. It also appeared that the insertions were scattered along its coding sequence and 3'-untranslated region (Figure 6), giving us reasonable chances of a gene knockout and maybe several mutant phenotypes with AtEP3 proteins truncated at different positions.

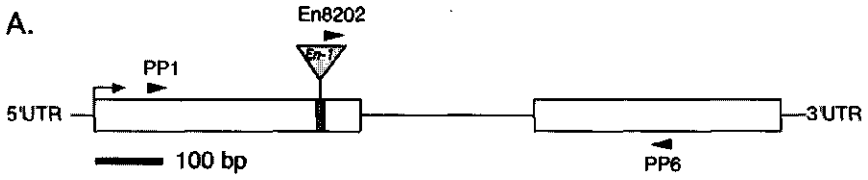
Genetic and phenotypic analysis of AtEP3 insertion lines

Seeds of the corresponding AMAZE lines were obtained from the Max-Planck Institüt für Züchtungsforchung (Köln, Germany) and plants were grown for analysis. Due to the presence of the transposase in the *En-1* element, both somatic and gametophytic insertions are unstable. Therefore the presence of the *En-1* insertion needs to be verified in each successive generation. The insertional alleles were first confirmed in each plant of each insertion line by PCR amplification and Southern blot analysis. To have an internal standard for DNA quality, two gene-specific primers were used simultaneously with one *En*-specific primer (Figure 7). This way, we also hoped to directly determine the genotype of the tested plant. However, due to the frequency of the excision events the wild-type allele, even in a plant homozygous for the insertion, was always amplified. By following the segregation of the *En-1* element inserted into *AtEP3* we were able to determine the insertion genotype of the mother plant for each line (Figure 7C).

The plants for which the insertion into *AtEP3* was confirmed were analyzed further at the morphological level and compared to wild-type *Columbia* plants as well as to plants of the same line without an insertion in the *AtEP3* gene. A broad range of morphological defects was observed together with an extensive variation between individual plants of the same line. This is most likely due to continuous transposon activity in the genome of these plants. The observed phenotypes ranged from high levels of anthocyanin, altered phyllotaxis, late flowering, very large or extremely small rosettes, no silique development to complete sterility, and were found in all possible combinations (Figure 8). None of these phenotypes segregated with the *En-1* insertion in *AtEP3* and therefore no direct link could be made with a disruption of the chitinase gene.

Based on the proposed role of AtEP3 during pollination and embryogenesis (Passarinho *et al.*, 2001) particular attention was paid to seed development, and siliques of all plants were analyzed in detail for seed abnormalities. Seed abortions were scored in dissected siliques of each plant and the results are summarized in Figure 9. Interestingly, some plants showed up to 100% seed abortion. The siliques were however fully developed, suggesting that the arrest in seed development was uncoupled from pod development. The seed abortion phenotype was highly variable within plants of the same line and even between siliques of the same plant. This variation was also qualitative, ranging from an early to a late arrest in seed development or both intermingled in the same silique. This phenotype was also found in plants that had no insertion in the *AtEP3* gene. However, the excision activity of the *En-1* element itself could also explain this situation. It remained therefore possible that the *En-1* element had excised

A.



B.

C.

Line	Segregation En-1	Genotype mother	% developed seedling
17F4	100%	Homozygous	100
29F2	3:1	Heterozygous	70
31A3	3:1	Heterozygous	100
37A3	100%	Homozygous	88
129C3	3:1	Heterozygous	76
Col.	-	-	94

Figure 7. Molecular analysis of the *AtEP3* insertion lines.
A. Position of the primers used for determining the presence of the *En-1* insertions in the *AtEP3* gene for each individual plant of the lines identified, as well as their genotype for the insertion. The example of line 31A3 is shown here. **B.** Example of an autoradiogram of the multiplex-PCR performed on genomic DNA from each plant of the individual line 31A3, after hybridization with an *AtEP3*-specific probe. The numbers above the lanes indicate the plant numbers of line 31A3 (e.g. 2 represents plant 31A3-2), **0** is the negative control of the PCR reaction (no DNA), and Col. is the positive control for the *AtEP3* primers (*Columbia* genomic DNA). When the *En-1* element is inserted in *AtEP3*, the lower band (*En-1*) is amplified and detected by the probe, the corresponding lane is marked **E**. The upper band (*AtEP3*) represents the wild-type *AtEP3* gene without insertion. Because of the presence of revertant sectors, both bands can be detected simultaneously, even in a plant homozygous for the insertion. On the other hand some plants gave no PCR product for *En-1* (noted -) indicating that the mother plant was heterozygous or that the *En-1* element has excised from its original position. Plants for which no PCR product was amplified, probably due to a DNA of bad quality, were not scored (noted ?). **C.** Segregation and genotype of the *En-1* insertion in the *AtEP3* gene for each individual positive line. The presence of the *En-1* element was verified per plant of each line by the PCR experiment described in A and B. The segregation ratios were determined by dividing the number of plants with the *En-1* insertion by the number of plants without. In the case of the line 31A3, shown in B, this was 10:3, i.e. ± 3:1. The genotype of the mother plant was deduced from these ratios. We also counted the percentage of seedlings that had developed 6 days after transfer to soil.

from the *AtEP3* gene in these lines but had left a footprint. Unfortunately, the seed abortion phenotype was absent in plants that still had an insertion into the *AtEP3* gene, suggesting that this phenotype is not linked to a disruption of the *AtEP3* gene. The detailed analysis of these plants was further complicated by the presence of multiple *En-1* insertions and by the high activity of the transposase that generated chimeric plants. To simplify the analysis, we decided to pursue with only one line and chose 31A3, which has an *En-1* element inserted in the first exon of *AtEP3*. In theory, this should provide us with the best opportunity of having a functional knockout. Some plants were back-crossed to *Columbia* in an attempt to eventually be able to analyze plants with a single *En-1* insertion in *AtEP3*. The progeny of those crosses and from selfed plants were grown and observed in detail. None of the already observed defects segregated in a Mendelian manner (data not shown). This reflected the instability of the various *En-1* elements present and confirmed that there was no phenotype at the plant level that could be linked to the disruption of the *AtEP3* gene.

Since the function of EP3 in carrot was only demonstrated during somatic embryogenesis (de Jong *et al.*, 1992) and supported by the expression pattern of AtEP3 in cultured cells (Passarinho *et al.*, 2001), we continued the analysis of line 31A3 by looking at somatic embryo development in some of its homozygous descendants. Embryogenic cultures were initiated in triplicate from dissected immature zygotic embryos from the *En-1* lines 31A3-7 and 31A3-10 and from *Columbia* as control. After three weeks, these cultures were scored for their embryogenicity. All cultures had only produced a few somatic embryos and rapidly became non-embryogenic (data not shown). No apparent difference between *En-1* lines and *Columbia* was observed. Medium was collected from these cultures and analyzed for the presence of the AtEP3 protein (Figure 10). The AtEP3 chitinase is only present in embryogenic cultures (Passarinho *et al.*, 2001) and although the embryogenic potential of wild-type cultures was rather low in these experiments, it was clear that the AtEP3 chitinase was produced and secreted at detectable levels in the medium. In the *En-1* cultures, AtEP3 was no longer detectable. This indicated that the disruption of the *AtEP3* gene indeed, as in lines 31A3-7 and 31A3-10, led to a complete absence of AtEP3 protein synthesis, or maybe to a truncated protein that is no longer recognized by the antibody. We conclude that absence of the AtEP3 protein does not lead to a visible developmental phenotype, neither at the plant level nor in embryogenic cultures.

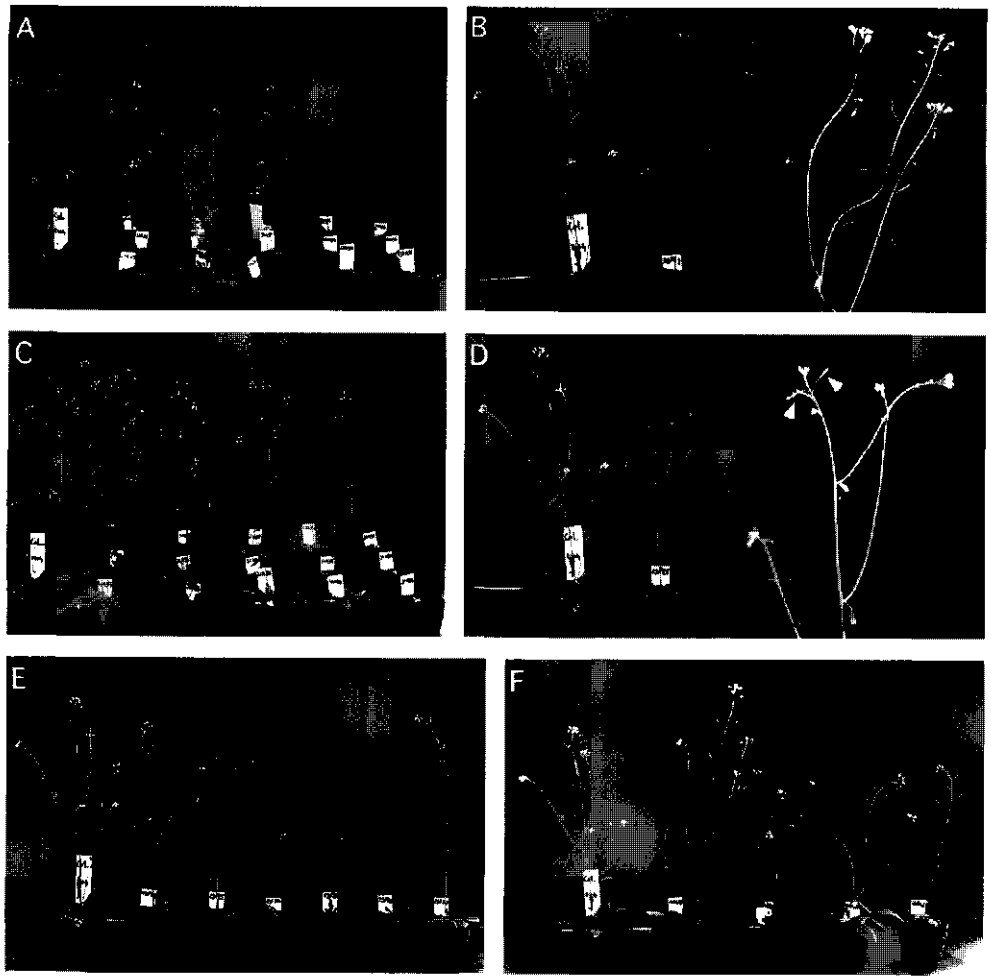


Figure 8. Examples of morphological defects observed in some *AtEP3* insertion lines.
A. Overview of plants from *AtEP3* insertion line 37A3 showing from right to left: tiny plants with small rosette and hardly any development, plants with a very large rosette and delayed development, plants with a single stem and a tiny rosette, plants with a very large rosette and a rather short shoot, and plants that are similar to the wild-type, which is in the last row from the right. **B.** Closer view of a sterile plant from line 37A3 compared to the wild-type on the left. The right panel shows a higher magnification of the non-elongating siliques found on the sterile plant. **C.** Overview of plants from *AtEP3* insertion line 31A3, showing some additional phenotypes such as plants (3rd row from the right) that are taller than the wild-type in the last row from the right. **D.** Closer view of a plant of line 29F2 having a delayed development as compared to the wild-type on the left. The first siliques are just appearing (arrowheads on the right panel) and the rosette leaves are smaller. **E-F.** Some of the phenotypes also seen in A and B are also present in lines 29F2 (E) and 17F4 (F) with a gradient in the severity of the defects (plant size and fertility in E, as well as branching (first plant from the right) and branching and rosette size in F). The wild-type is the last plant from the right in both panels.

Discussion

The aim of this work was to obtain a knockout mutation in the *Arabidopsis AtEP3* endochitinase gene. We have successfully screened two *Arabidopsis* plant populations generated by insertional mutagenesis, representing over 80,000 insertions. In total, we have identified six insertions in the *AtEP3* gene and determined their exact position by sequencing. A T-DNA insertion was located at the start of the second exon, corresponding to the catalytic domain of the enzyme, and this should have resulted in a truncated non-functional protein. However, the individual insertion line could not be identified, most likely due to a problem in the pooling procedure. In addition, one *En-1* insertion was found in the first exon, three in the second and a final one in the 3'-untranslated region. All corresponding individual insertion lines were identified and a detailed phenotypic analysis was performed. Multiple *En-1* insertions are present in these plants, and they are somatically and germinally unstable. This may have caused the observed variety of phenotypes. Putative AtEP3 mutants were analyzed in detail for morphological defects during plant development that could be linked to an insertion in the *AtEP3* gene. The main focus was on seed development and somatic embryogenesis in view of the expression pattern of the *AtEP3* gene (Passarinho *et al.*, 2001). Although in one *En-1* insertion line we could show that the AtEP3 protein was no longer produced, it appeared that none of the observed phenotypes could be genetically linked to the disruption of the *AtEP3*gene.

In carrot, the EP3 endochitinase was identified by its ability to restore embryo development in the mutant cell line *ts11* (de Jong *et al.*, 1992). Later studies revealed that it is not only the absence of the EP3 protein, but presumably also the absence of a related class I chitinase that made *ts11* respond to the addition of EP3 protein (de Jong *et al.*, 1995; Kragh *et al.*, 1996). Indeed, *ts11* also responded to this class I chitinase by producing more embryos (de Jong *et al.*, 1995). The phenotype of the *ts11* mutant line is thought to be the result of a secretory defect rather than a mutation in the carrot *EP3* chitinase gene (Baldan *et al.*, 1997). Recent results (van Hengel *et al.*, 2001) showed that this chitinase had a stimulatory effect on somatic embryogenesis from carrot wild-type protoplasts, suggesting that the effect of chitinases on embryogenesis was not restricted to *ts11*. Therefore, it could well be that without disrupting other *Arabidopsis* chitinase genes, we will not be able to see any somatic embryo phenotype. Based on these observations we did not pursue the further analysis of these insertion lines, neither at the molecular (mRNA quantification) nor at the genetic (back-crossing) level.

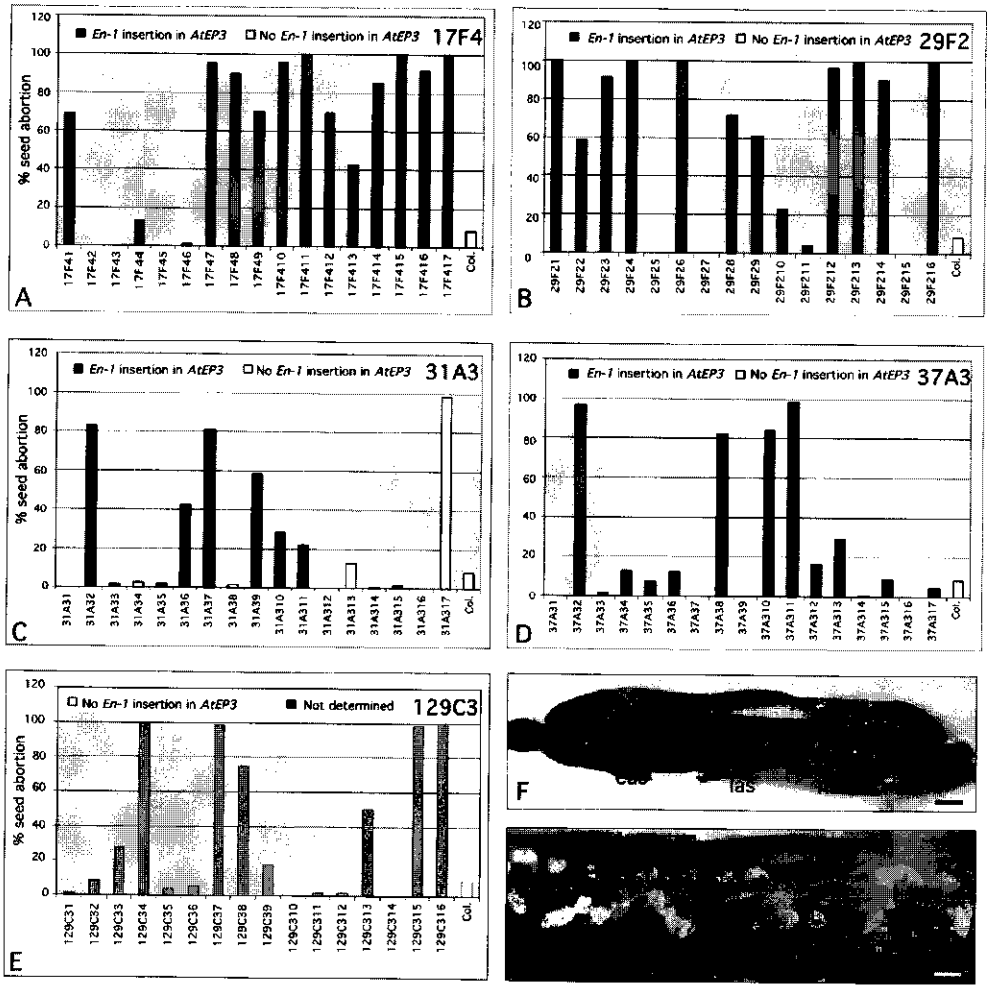


Figure 9. Phenotypic analysis of seed development in the *AtEP3* insertion lines.
A-E. Distribution of seed abortions per plant of each line containing an *En-1* element in the *AtEP3* gene. Values on the Y-axis represent the percentage of total seed abortions per plant. The plant numbers are indicated on the X-axis. *Columbia* (Col.) is the wild-type used for comparison with each line. Plants without a percentage of seed abortion recorded were fully sterile and did not produce any silique. **F.** Example of a silique with nearly 100% seed abortion. Only two normal seeds (ns) are visible. The aborted seeds show either early abortions (eas) and are small and white or show late abortions (las) and are shrunken and brown. Bar = 12 mm. G. Higher magnification of early (eas) and late aborted seeds (las). Bar = 3 mm.

The efficient screening of large plant collections depends largely on the pooling strategy employed that must allow the analysis by PCR of thousands of individual plants simultaneously for a rapid identification of an individual plant. Extreme care must be taken in this operation since, as we experienced, a small mistake can be fatal for the identification of

an individual insertion line. Three-dimensional pooling of the DNA of these plants, such as performed for the AMAZE collection, has proven to be a very effective method. Another limiting factor to consider in this approach is the nature of the inserted element. T-DNA insertions are stable and thereby allow an easier molecular and genetic analysis of mutant phenotypes. However, their integration into the plant genome is often the cause of truncations and local rearrangements after which only one of the T-DNA borders is recovered (Krysan *et al.*, 1996). In addition T-DNA insertions often consist of several copies inserted in tandem, all of which can complicate their detection in a gene of interest. Another disadvantage is that their insertion frequency is low (1-2 loci per line, (Bouchez and Hofte, 1998)), making it more difficult to achieve genome saturation. Indeed, one needs to generate very large plant populations, considering that 120,000 independent insertions are estimated to be necessary for having 95% chance of hitting any gene at least once (Bouchez and Hofte, 1998).

Transposon mutagenesis allows the generation of a large number of insertions in a relatively small population of plants. Depending on the system used, up to 30 transposon copies can be present in the genome of a particular line (Speulman *et al.*, 1999), which makes it easier to identify insertions in a given gene. However, this large number of insertions can also significantly complicate the phenotypic analysis and be very time consuming. Indeed, such plants often have multiple phenotypes, linked or not, and it can be difficult to associate them with a particular insertion. This requires an extensive genetic and molecular analysis on large progenies with several generations of back-crossing involved in order to reduce the number of insertions to the one in the gene of interest. In the AMAZE collection for instance, the transposon is autonomous and is active both somatically and germinally. Consequently, one needs to "stabilize" the insertion of interest and this cannot be done, as in two-component systems, by crossing out the transposase source (Speulman *et al.*, 1999; Tissier *et al.*, 1999).

One way out is to screen for lines with excised elements that left a footprint behind in the gene of interest and that have no other insertion in their genome, which is particularly time- and labor-consuming. This implies successive back-crosses to eliminate all other mobile elements and systematic molecular confirmation of the presence/absence of the transposon in each plant at each generation. The identification of a footprint requires then systematic amplification, cloning and sequencing of the locus of interest. In two-component transposon systems this analysis is facilitated by the stability of the insertion and the presence of a selection marker in the transposon construct (Altmann *et al.*, 1995; Speulman *et al.*, 1999; Tissier *et al.*, 1999). Transposon mutagenesis also allows the remobilization of the transposable element in order to determine if reversion of the observed phenotypes occurs, to

confirm their link with the analyzed insertion. In the case of T-DNA mutagenesis, this requires the introduction of a wild-type allele for complementation of the mutant phenotype.

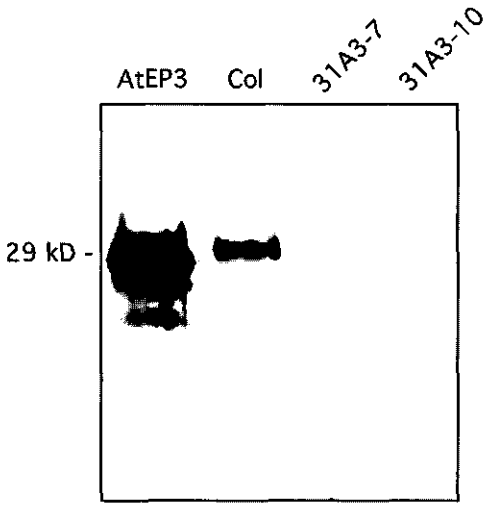


Figure 10. Western-blot analysis of AtEP3 protein production in somatic embryogenic cultures of *AtEP3* insertion lines.
Equal amounts of proteins isolated from the medium of embryogenic cultures of *Columbia* (Col.) and two homozygous 31A3 lines (31A3-7 and 31A3-10) were analyzed by Western-blotting, using an antibody raised against the carrot EP3 protein (de Jong *et al.*, 1995). In the lane marked AtEP3, a sample of an in vitro-produced AtEP3 protein (Passarinho *et al.*, 2001) is shown as the positive control for the immunodetection.

A more general problem associated with both methods is the frequently observed absence of a phenotype for an insertion in the gene of interest. It is indeed estimated that only 1-5% of the insertions do display mutant phenotypes (Pereira, 2000). The reason often proposed is genetic redundancy. Many genes belong to families that are the result of gene duplications(The Arabidopsis Genome Initiative, 2000) and of which the members can have a similar function, thereby masking any possible phenotype. This is clearly demonstrated in the work of Meissner and coworkers (Meissner *et al.*, 1999) who showed that individual knock-out of 36 members of the R2R3 *MYB* gene family does not give rise to a visible morphological phenotype. This genetic redundancy could also account for the absence of a phenotype in plants without a functional AtEP3 protein, since six other class IV chitinase genes and a related class I chitinase gene are known to be present in the *Arabidopsis* genome (Passarinho *et al.*, 2001).

Another cause for seemingly redundant functions is a failure to spot the actual phenotype under the screening conditions applied. In several cases, growing the putative mutant plants in a variety of environments appeared to be essential for revealing their phenotypes (Geelen *et al.*, 2000; Hirsch *et al.*, 1998; Meissner *et al.*, 1999). When there is no clear hint on what type of phenotype can be expected, this can be a difficult task. In the case

of AtEP3, based on its expression pattern one could envisage defects in pollen development, anther dehiscence, pollen germination and/or pollen tube growth, all leading to male sterility. We could also expect a root or root hair phenotype or an effect on somatic embryo development. Since none of these was apparent or linked to the absence of AtEP3 protein, it remains uncertain which function this chitinase has in *Arabidopsis*. Nevertheless, this is the first report, to our knowledge, of a reverse genetics approach to study the role of a chitinase gene during plant development and it clearly reveals some of the limitations of these strategies, principally when dealing with gene families.

Systematic large-scale sequencing of insertion sites should allow the identification in silico of knockout mutants per family, saving a substantial amount of work (*Arabidopsis* Sequenced Inserts at http://ukcrop.net/agr/insert.html). Subsequent crosses could then be immediately initiated to generate multiple mutants. The *Arabidopsis* Knockout Facility developed at the University of Madison (Sussman *et al.*, 2000) also aims at improving this methodology e.g. using the concept of "launching pads" to create double mutants in one go. New methods are continuously under development. For example Targeting Induced Local Lesions IN Genomes (TILLING, McCallum *et al.* (2000) allows the identification of chemically induced mutations in target sequences, combining the efficiency of Ethyl Methane Sulfonate (EMS) mutagenesis with the ability to automatically detect Single Nucleotide Polymorphisms (SNPs) by denaturating high-performance liquid chromatography (DHPLC, Steinmetz *et al.* (2000).

Materials and Methods

Plant growth conditions

Arabidopsis thaliana (L.) Heynh plants were grown in soil under long day conditions (16h photoperiod, 22°C). Seeds were germinated on wet filter paper and transferred to soil after 2 days of vernalization at 4°C.

Plant collections

The Versailles T-DNA lines were generated by vacuum infiltration of the ecotype *Wassilewskija* (WS) with the pGBK5 binary vector (Bechtold *et al.*, 1993; Bouchez *et al.*, 1993). Genomic DNA of each individual line was pooled in primary pools (PPs), each containing 48 individual lines, these were gathered into superpools (SPs) of 8 PPs, and 29

hyperpools (HPs) were formed of 2 SPs each. A total of 22,176 lines were screened, representing approximately 33,000 insertions.

The AMAZE lines were generated in the ecotype *Columbia* (Col.), as described in (Baumann *et al.*, 1998). They were organized in four dimensions, allowing each individual line to be designated according to its three-tray, single tray, row and column coordinates (Baumann *et al.*, 1998). Subpopulations I and II were screened representing a total of 8,000 lines carrying approximately 48,000 *En-1* insertions.

Screening of the Versailles T-DNA collection

The PCR screening was performed with *AtEP3* gene-specific primers designed according to INRA's recommendations and tested in the screening conditions described hereafter. Because T-DNA insertions often have only one intact border (Nacry *et al.*, 1998), primers for both ends of the T-DNA were used in combination with the selected gene-specific primers. The primary screenings were performed at INRA (Versailles, France) on the 29 HPs and the primer pair giving the best amplification was used further. The PCR reactions were performed using 25 pmol of each primer, 0.2 mM dNTPs, and 1 unit of Taq Polymerase (Boerhinger Manheim, Germany) in 10 mM Tris-HCl pH 9.0, 50 mM KCl, 0.1% (v/v) Triton X-100 and 2.5 mM $MgCl_2$. The cycle parameters were the following: 5 min at 94°C; 10 cycles of 1 min at 94°C, 1 min at 65°C (minus 1°C per cycle), 2 min at 72°C; 40 cycles of 1 min at 94°C, 1 min at 55°C and 2 min at 72°C followed by 5 min at 72°C. PCR products were analyzed by agarose gel electrophoresis and transferred by alkali blotting onto two Hybond-N+ (Amersham, UK) membranes simultaneously. Each membrane was hybridized separately to a gene-specific probe (*AtEP3* genomic sequence) and a T-DNA probe (mixture of Left and Right Border sequences). Only bands hybridizing with both probes were considered as being positive and the corresponding HPs were analyzed in the very same way at the SP level and so forth until the identification of individual lines carrying a T-DNA insertion in the *AtEP3* gene. The site of insertion was confirmed by nested PCR and sequencing of the obtained PCR products.

Screening of the AMAZE collection

Each tested *AtEP3* gene-specific primer was used in combination with the *En-1*-specific primers En205 (*En* 5' end) and En8130 or En8202 (*En* 3' end, Figure 1C). The PCR reactions were performed using 20 pmol of each primer, 50 µM dNTPs, and 1.25 units of Taq Polymerase (Boerhinger Manheim, Germany) in 10 mM Tris-HCl pH 9.0, 50 mM KCl, 0.1%

(v/v) Triton X-100 and 2.5 mM $MgCl_2$. The cycle parameters were the following: 2 min at 85°C; 40 cycles of 40 sec at 94°C, 1 min at 65°C and 2 min at 72°C; followed by 5 min at 72°C. PCR products were analyzed by agarose gel electrophoresis and transferred by alkali blotting onto Hybond-N+ (Amersham, UK). The produced membranes were hybridized with a radiolabeled *AtEP3* cDNA probe and samples giving hybridizing PCR products for each pooling dimension were considered as positives, their coordinates corresponding to the individual lines with an insertion into the *AtEP3* gene. Insertion sites were confirmed by nested PCR and sequencing of the obtained PCR products.

Morphological screening

Plants from *En-1* lines with and without insertion in the *AtEP3* gene were grown in parallel with *Columbia* wild-type plants and visually monitored for morphological defects such as leaf shape and number, phyllotaxis and fertility. Seed set was analyzed by collecting about 10 siliques per plant starting from the fourth silique below the terminal inflorescence. Siliques were opened under a Nikon SMZ-2T binocular microscope and seeds were counted and classified according to their stage of development: early aborted (white and tiny), late aborted (brown and shrunken) and fully developed. Percentages of seed abortion per silique and per plant (total early plus late) were then determined.

In vitro culture

Arabidopsis embryogenic cultures were initiated from dissected immature zygotic embryos of the ecotype Col. and AMAZE lines homozygous for an *En-1* insertion in the *AtEP3* gene and maintained as described by (Mordhorst *et al.*, 1998).

Protein purification, and Western blotting

Total protein extracts from *Arabidopsis* embryogenic cell suspension culture media were obtained as described by (van Hengel *et al.*, 1998), separated by SDS-PAGE according to (Laemmli, 1970) and subsequently transferred to an Immobilon™-P PVDF Transfer Membrane (Millipore, Bedford, MA, USA). Immunological detection was performed as described by (de Jong *et al.*, 1995) with a rabbit antiserum raised against carrot EP3.

Acknowledgements

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Chapter 5

Functional analysis of the *Arabidopsis* AtEP3 endochitinase: overexpression and antisense approaches

Abstract. Plant endochitinases (EC 3.2.1.14) play multiple roles in defense and stress responses as well as in development. It is therefore essential to first determine the role of individual chitinases in order to understand how they can be involved in such different processes. Based on its expression pattern, the *Arabidopsis* AtEP3 endochitinase may be involved in several developmental processes. We have generated transgenic plants with altered levels of AtEP3 chitinase mRNA and studied the effect of these changes on the development of *Arabidopsis* plants. Overexpression of the *AtEP3* gene did not result in any visible morphological change whereas down-regulation revealed defects in seed set and root hair development. An additional mutant, for which we could not establish a link with a change in AtEP3 expression was identified. This mutant showed a male sterility phenotype as well as a defect in root hair development. Functional implications of the observed phenotypes are discussed in relation with the AtEP3 chitinase.

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Introduction

Endochitinases (EC 3.2.1.14) are abundant hydrolases widely found in higher plants (Graham and Sticklen, 1994). Based on their primary structure, endochitinases are organized in five different classes numbered from I to V (Neuhaus *et al.*, 1996). Endochitinases are often qualified as pathogenesis-related (PR) proteins since the genes encoding some, but not all, chitinases can be induced by various elicitors of fungal, bacterial and viral origin. They have been proposed as potential fungal growth inhibitors (Schlumbaum *et al.*, 1986) as some endochitinases are capable, especially in association with β-1,3-glucanase (EC 3.2.1.39), of degrading fungal cell walls and inhibiting hyphal tip growth (Mauch *et al.*, 1988). Class III chitinases exhibit lysozymal activity and are therefore believed to be involved in resistance to bacteria (Majeau *et al.*, 1990). However, endochitinases are also induced in more general stress reactions such as wounding and application of salicylic acid or heavy metal salts, as well as after application of growth regulators such as ethylene, auxins and cytokinins (reviewed in Graham and Sticklen, 1994).

There are also reports of chitinases, of which the expression appears to be developmentally regulated, e.g. at specific stages in organs of tobacco flowers (Neale *et al.*, 1990; Trudel *et al.*, 1989) suggesting that chitinases could also play a role in developmental processes such as pollination (Leung, 1992). But the first functional evidence came from work done on a carrot mutant cell line, from which arrested somatic embryos could be rescued by the EP3 endochitinase, a member of the class IV chitinases (de Jong *et al.*, 1992). Interestingly the same effect could be mimicked by certain bacterial lipo-chitooligosaccharide signals (Nod factors) involved in the *Rhizobium*-legume symbiosis (de Jong *et al.*, 1993), indicating that similar N-acetylglucosamine (GlcNAc)-containing signal molecules could be present in plants. This led to the hypothesis that such hypothetical molecules require processing by chitinases to induce or control developmental processes such as embryo development. More recent studies tend to support this hypothesis by showing that plant chitinases are involved in the processing of Nod factors required for root nodule formation (Ovtsyna *et al.*, 2000); (Schultze *et al.*, 1998), suggesting that Nod factors are natural substrates for some legume chitinases. However, to date no plant-derived Nod analogue has been identified. Recently, evidence of an endogenous plant substrate for endochitinases was obtained by van Hengel *et al.* (2001), who showed that certain classes of arabinogalactan proteins (AGPs), present in carrot embryogenic cultures and seeds, contain GlcNAc residues. While these AGPs were active in promoting embryo formation from carrot protoplasts, after processing by the EP3 chitinase their activity on embryo formation was enhanced. These observations suggest that

endogenous substrates for plant chitinases do exist and that they have a biological activity that can be enhanced by chitinase treatment (van Hengel *et al.*, 2001).

In order to eventually provide a genetic background to analyze these mechanisms, we have decided to study the *Arabidopsis* ortholog of the carrot EP3 chitinase (Passarinho *et al.*, 2001). The expression pattern of the *Arabidopsis* gene was found to be highly regulated and suggested that it might be involved in development but as also proposed in carrot (van Hengel *et al.*, 1998), this role is probably not restricted to somatic embryogenesis. Based on this work, AtEP3 could be involved in pollen maturation, germination and/or pollen tube growth as well as root hair elongation. Expression in non-dividing tissues (i.e. leaf hydathodes and stipules) suggested a possible role in programmed cell death (PCD). Although difficult to determine, solely based on expression studies, it was argued that a role in PCD might be at the heart of the different functions of the AtEP3 chitinase (Passarinho *et al.*, 2001).

In this work, we addressed the possible function(s) of this chitinase by generating transgenic *Arabidopsis* plants transformed with the *AtEP3* cDNA in sense and antisense orientation under the control of the strong cauliflower mosaic virus 35S RNA (CaMV 35S) promoter. Transformants were analyzed for changes in *AtEP3* gene expression and phenotypes correlated with such changes.

Results

Transformant selection

We have generated a number of transgenic lines in an attempt to affect the AtEP3 endochitinase level by means of overexpression, co-suppression or antisense suppression. Two constructs were designed for this purpose (Figure 1A and B) and introduced into the genome of *Arabidopsis* wild-type plants by using *Agrobacterium*-mediated transformation. Transformants were selected on the basis of their resistance to kanamycin conferred by the presence of the neomycin phosphotransferase II (NPTII) gene in the introduced construct. The progeny of the selected transgenic plants subsequently underwent a number of analyses as summarized in Table 1.

Plants transformed with the overexpression construct *Pe35S::AtEP3* yielded a sufficient number of transformants, whereas transformation with the antisense construct *Pe35S::α-AtEP3* resulted in only three transformants. However, this was due to technical difficulties rather than to the construct used. Seeds of all individual transformants were harvested and selected as the T_1 seeds of their mother plant on germination medium with

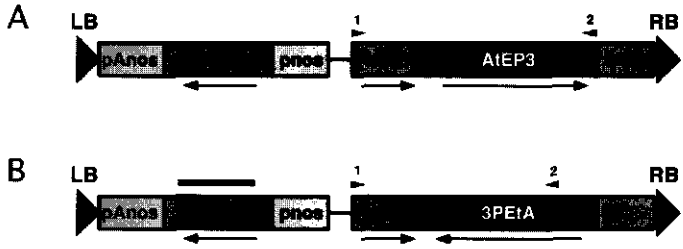


Figure 1. Schematic representation of the generated T-DNA constructs and DNA analysis of the transformed plants.
The arrows under the *AtEP3* and *NPTII* cDNAs indicate the orientation of the coding sequence and the arrow under the enhanced CaMV 35S promoter (Pe35S) indicates the direction of the transcription **A.** AtEP3 overexpression construct *Pe35S::AtEP3*. The arrowheads (1) and (2) indicate the position of the primers used for the PCR analysis, 35S-5' and TAI2, respectively. **B.** AtEP3 antisense construct *Pe35S::α-AtEP3*. The arrowheads (1) and (2) indicate the position of the primers 35S-5' and PP5, respectively. The bar above the *NPTII* gene represents the fragment used as a probe for the Southern blot analysis (Figure 5A).

kanamycin. T_2 and T_3 resistant plants were analyzed at the DNA level to confirm the actual presence of the construct and to verify that the *Pe35S::AtEP3* and the *Pe35S::α-AtEP3* cassettes were intact. This was done by PCR using specific primers present in this part of the transgene only (Figure 1A and B). The result of the kanamycin selection was confirmed for all plants tested (Table 2) and this also showed that at least one copy of the introduced transgene was intact in all selected plants.

Transgene	T_1 Lines	Segregation analysis [a]	DNA analysis [b]		RT-PCR	Seed set [c]	Pollen [d]	S.E. [e]	Root [f]
			Southern	PCR					
Pe35S::AtEP3	52	38	nd	38	12	21	nd	9	9
Pe35S::a-AtEP3	3	3	3	3	3	3	3	2	3

Table 1. Analyses performed on AtEP3 overexpression and antisense lines.
T_1 lines are the number of independent transformants obtained for each transgene. Each column indicates the number of families descending from the T_1 lines on which the analyses were performed.
[a] The segregation analysis was carried out on the basis of resistance to Kanamycin. [b] The DNA analysis consisted of determining the presence of the transgene (PCR) in the plants analyzed and the number of copies inserted per genome (genomic Southern blotting). Expression levels of the *AtEP3* gene were determined by gene-specific RT-PCR. [c] Determination of the number of normal seeds formed per silique and per plant. [d] Pollen in vitro germination. [e] Seeds or dissected immature zygotic embryos of these lines were tested for their ability to form somatic embryos. [f] The roots of seedlings from these lines were analyzed for morphological defects.

Line	T_2 Kan^R : Kan^S	Construct presence	T_3 Kan^R : Kan^S	T_4 Kan^R : Kan^S	Copy number
C4	1:1	+	C4-1 / 0:1	nd	nd
			C4-2 / 0:1	nd	
			C4-3 / 3:1	nd	
			C4-4 / 1:6	nd	
			C4-5 / 1:1	nd	
D3	1:1	+	D3-1 / 8:1	nd	>1
			D3-2 / 6:1	nd	
			D3-3 / 2:1	nd	
			D3-4 / 8:1	nd	
			D3-5 / 1:2	nd	
			D3-6 / 1:0	nd	
			D3-8 / 33:1	nd	
D8	1:1	+	D8-1 / 15:1	nd	>1
			D8-2 / 1:0	nd	
			D8-3 / 1:0	nd	
			D8-4 / 1:0	nd	
			D8-5 / 2:1	nd	
			D8-6 / 1:0	nd	
			D8-7 / 1:0	nd	
			D8-8 / 1:1	nd	

Line	T_2 Kan^R : Kan^S	Construct presence	T_3 Kan^R : Kan^S	T_4 Kan^R : Kan^S	Copy number
B1	16:1	+	B1-1 to B1-7 / <1	-	1 to 5
			B1-8 / 3:1	3:1 and 1:0	
			B1-9 / 0	-	
			B1-10 / 4:1	-	
			B1-13 / 3:1	-	
			B1-16 / 1:0	1:0	
			B1-17 / 1:0	1:0	
			B1-23 / 2:1	-	
B2	5:1	+	B2-1 / 2:1		5
			B2-2 / 1:3		
			B2-3 and B2-4 / 3:1		
			B2-5 / 8:1		
			B2-9 to B2-22 / <1		
B3	3:1	+	3:1	3:1	1

Table 2. Summary of transgene segregation analysis on the *Pe35S::AtEP3* and *Pe35S::α-AtEP3* lines.
The transgene segregation was followed by scoring seedling resistance to kanamycin and is indicated by the Kan^R:Kan^S ratio (kanamycin resistant over kanamycin sensitive seedlings). This analysis was performed on the progenies of individual T_1 transformants, which names are indicated in the first column (see text for nomenclature). The left part of the table shows the results of *Pe35S::AtEP3* lines that display phenotypes whereas the right part shows the results of the *Pe35S::α-AtEP3* lines. The third column indicates that the presence of the transgene was confirmed by PCR analysis. In the fourth column the ratios obtained follow the name of the line. The copy number value is estimated from the Kan^R:Kan^S ratios for the *Pe35S::AtEP3* lines and is based on the Southern blot analysis shown in Figure 5A for the *Pe35S::α-AtEP3* lines.

Segregation of the transgene was analyzed by scoring the ratio between seedlings that are resistant and seedlings that are sensitivity to kanamycin. Table 2 only shows the results obtained with lines that in the subsequent analyses displayed possible phenotypes. The line nomenclature used through this manuscript is as follows. The capital letter denotes the transformed plant (T_0), the number that follows represents a T_1 line descendant, an additional number separated by a dash is added at each subsequent generation. For example, B1-8-18 is the 18th T_3 line descending from the 8th T_2 line descending from T_1 line B1, itself originating from transformed plant B.

The main conclusion from the kanamycin selection is that a great variability was observed among the T_1 lines but also and mainly among their descendants. This is probably the result of multiple insertions as suggested by ratios of kanamycin resistance over kanamycin sensitivity (Kan^R:Kan^S) that were above the 3:1 value expected in case of a single transgene insertion. A large number of lines presented such high ratios, especially visible at the T_3 generation where it was sometimes accompanied by a loss of kanamycin resistance. This is illustrated in Table 2 by the results of the descendants of the T_1 lines C4, D3 and D8 transformed with the overexpression construct, and T_1 lines B1 and B2, transformed with the antisense construct. The occurrence of multiple insertions of the transgene was shown by Southern blot analysis on some of the lines (Figure 5). Seeds of descendants such as C4-1 and C4-2 became totally sensitive to kanamycin (0) whereas those of descendants such as C4-4 gave inverted Kan^R:Kan^S ratios (<1), with a larger number of seedlings that are sensitive to kanamycin as compared to the seedlings that are resistant. These "newly" sensitive seedlings often showed less severe phenotypes, being slightly bigger than fully sensitive seedlings and their cotyledons showing patches of green, also indicative of partial resistance. However when transferred to soil most of the partially resistant seedlings died and in the subsequent generation only normal Mendelian segregation ratios were found (B1 and B2 lines).

Some ratios indicative of transmission defects were also found especially in lines with the *Pe35S::AtEP3* construct. The lines presented here (C4, D3 and D8) indicate 1:1 ratios at the T_2 generation suggesting a gametophytic lethal phenotype that allows complete seed development only if the transgene is transmitted by one of the gametes but not by the other. However, at the T_3 generation we can see that only C4-5 and D8-8 still segregate 1:1. The other descendants are homozygous (e.g. D3-6 or D8-6) or show different segregation ratios such as D3-1 (8:1) or D8-1 (15:1). Therefore, based on the variations observed from the T_2 to the T_3 generation, it is not very likely that these Kan^R:Kan^S ratios are the result of actual transmission defects. They most likely reflect artifacts of the kanamycin selection.

Another remarkable observation concerned antisense line B3 and its progeny. This is the only line in which the transgene segregated as a single insertion giving a 3:1 ratio. However, this ratio remained the same in all subsequent generations. Seeds always seemed to come from heterozygous plants, since they were never 100% resistant to kanamycin. A closer look at this line revealed that one third of the kanamycin-resistant descendants did not produce any seeds. This suggests that homozygous plants would be sterile as a result of a recessive sporophytic effect of the transgene.

Analysis of the AtEP3 expression level in the Pe35S::AtEP3 and Pe35S::α-AtEP3 transformants

A first step of our analysis was to determine the effect of the transgenes on the expression of the *AtEP3* gene. RNA was isolated from seedlings and inflorescences of individual lines of each family and gene-specific RT-PCRs were performed on these samples. The *AtEP3* mRNA levels were compared for each line with the wild-type using the cyclophilin gene *ROC5* (Chou and Gasser, 1997) as an internal standard (Figure 2). During this analysis we encountered a number of difficulties mostly due to the low expression level of the *AtEP3* gene in wild-type plants. This hindered quantification in the case of expression levels that may have been lower than the wild-type for which the detection was limited by the number of PCR cycles used to stay in the linear range of the reaction from our internal standard.

In the case of the *Pe35S::AtEP3* lines, the analysis was reasonably straightforward and clearly showed that the *AtEP3* gene was overexpressed in most of the lines, with relative expression levels reaching almost 7 times the level of the wild-type (Figure 2A and B). These levels also varied quite a lot within individuals of a family (see D19 and D8 samples). Lines D8-4 and D8-5 on the other hand showed expression levels comparable to the wild-type and only one line, C4-5, showed an approximately 10-fold decrease in expression.

In the case of the *Pe35S::α-AtEP3* lines, the analysis was somewhat more complicated since our RT-PCR experiments were not designed to distinguish between endogenous *AtEP3* mRNA and the antisense mRNA. When using *AtEP3* gene-specific primers that amplified both transgene and endogenous mRNAs, often the relative expression levels were found to be higher in the transgenic plants than in the wild-type. We interpreted this as a manifestation of the transgene expression in all cells of the tissue used for RNA isolation. The expected antisense suppression is likely to occur only in cells expressing both the endogenous gene and

the transgene simultaneously. The expression pattern of the *AtEP3* gene is very restricted (Passarinho *et al.*, 2001) and represents only a minor fraction of the cells present in the tissues we analyzed, i.e. pollen grains versus complete inflorescences or root hairs versus complete seedlings. To overcome these potential drawbacks we undertook several approaches. (i) RNA was extracted from isolated pollen grains and (ii) a primer was designed to be specific for the 3'untranslated region of the endogenous gene upstream the polyadenylation signal in order to rule out amplification of the cDNA reverse-transcribed from the transgene mRNA. Both approaches failed to provide a reliable quantification. The variations between individual experiments, especially with pollen RNA due to the low amounts of starting material, were simply too high. Consequently, we did not succeed to determine any change in AtEP3 expression in the antisense lines.

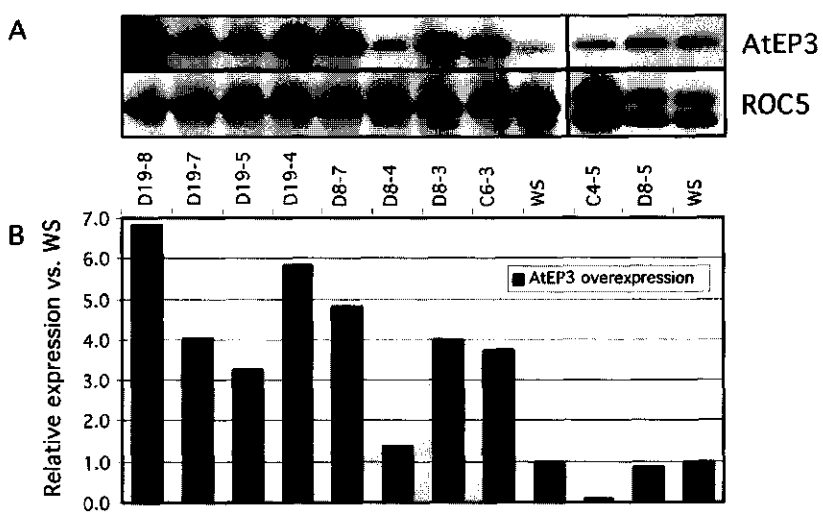


Figure 2. RT-PCR analysis of *AtEP3* gene expression in *Pe35S::AtEP3* plants.
A. Autoradiogram of an RT-PCR performed on RNA from seedlings of *Pe35S::AtEP3* lines. The upper panel shows the result for the *AtEP3* mRNA and the lower the result for the *ROC5* mRNA after hybridization of the PCR products with a radiolabeled gene-specific probe. The sample names/plant codes are indicated under the lanes using the following nomenclature. The capital letter represents the plant transformed with the construct and the number that follows stands for the T_1 descendant. For T_2 descendants this is followed by an additional number separated by a dash and for T_3 lines a third number is added and so forth. For example D19-8 is the 8th T_2 line descending from the T_1 line D19. WS is the wild-type. **B.** Quantification of the hybridization signals from panel A. Hybridization signals were quantified using the software ImageQuant® and the *AtEP3* values were corrected according to the values obtained with *ROC5*. These values were then expressed as a ratio (corrected signal intensity for transgenic plant ÷ corrected intensity for WS) representing the relative expression level of *AtEP3* in the transgenic plant as compared to the wild-type and plotted as an histogram with the sample names above the bars.

Analysis of seed formation in the Pe35S::AtEP3 and Pe35S::α-AtEP3 transformants

In parallel to the analysis of the *AtEP3* mRNA levels we conducted a phenotypic analysis on the selected transformants. Based on the supposed role of the AtEP3 chitinase during embryo development, we began by looking for defects in seed formation. Siliques of individual plants were analyzed for a number of transgenic T_2 lines (Table 1). Most lines presented no difference with the wild-type and only the results of the lines for which we did find a difference are shown in Table 3.

Line	Normal seeds	Late abortions	Early abortions	Total abortions	Total seeds	% abortions
C4-5	385	2	265	267	652	40.9
D8-5	518	0	218	218	736	29.6
D8-6	225	2	94	96	321	29.9
D3-8	336	1	204	205	541	37.9
B1	320	3	256	259	579	44.7
B2	716	11	461	472	1188	39.7
B3	1167	23	1562	1585	2752	57.6
WS	607	7	74	81	688	11.8

Table 3. Seed set analysis of the *Pe35S::AtEP3* and *Pe35S::α-AtEP3* lines.
This table shows an example of the results we obtained while observing seed formation in immature siliques. Only *Pe35S::AtEP3* lines differing from the wild-type, WS, are shown here. D8-5 and D8-6 are T_2 lines descending from the same T_1 line D8. The results of the *Pe35S::α-AtEP3* lines consist of the observations made on several T2 heterozygous descendants of each of the three T_1 lines.

A first conclusion to be drawn is that the transmission defects suggested by the kanamycin selection may be found back in this analysis. Indeed, in lines where the seed set is affected, the level of seed abortion is about twice that of the wild-type. This could be in agreement with the 1:1 Kan^R:Kan^S ratios shown in Table 2. Although, based on the results of the kanamycin selection this would only be true for line C4-5. However, we cannot rule out a genetic discrepancy between the actual transmission of the transgene and the observed resistance to kanamycin. The level of resistance may indeed depend on the location and number of transgene insertions. The second conclusion concerns the type of abortions observed. They consist in great majority of early arrests, while late arrests are only found at the level seen in the wild-type (± 1%). The early arrests we observed can be due to ovule or pollination defects. Based on the expression pattern of the *AtEP3* gene in developing pollen (Passarinho *et al.*, 2001), the latter possibility is more likely. A lower or higher expression of the gene in pollen grains could indeed disturb pollen germination and or pollen tube growth and consequently

affect fertilization and seed development.

In antisense line B3, the level of seed abortion is above 50% in heterozygous plants whereas in homozygous plants, there is no silique elongation. However some escapes do occur and were noticed when harvesting the dried sterile plants. 24 plants were put into individual seed bags and on average 5.38 ± 4.95 seeds were found per plant, indicating that fertilization can still occur in these plants. Some of the harvested seeds were sown and all plants appeared to be sterile (data not shown), confirming that the B3 sterile plants are homozygous. This is the only line where such a phenotype could be observed.

Interestingly, line C4-5 that exhibits a strongly reduced seed set (Table 3) also shows a 10-fold decrease in *AtEP3* mRNA level (Figure 2). However, lines D3-8 or D8-6 that have a 2 and 3-fold increase, respectively, of AtEP3 expression as compared to the wild-type (data not shown), show a very similar phenotype. In addition, lines with the highest expression levels (D19 family, Figure 2), do not show any defect in seed set (data not shown) and lines with expression levels comparable to the wild-type (e.g. D8-5, Figure 2) do show a seed abortion phenotype. Furthermore, our failure to quantify the *AtEP3* mRNA levels in the antisense lines does not allow us to conclude whether the observed defects in the lines B1, B2 and B3 could be due to a reduction in AtEP3 expression. Nevertheless, based on the variations found in these results we can assume that a direct correlation between the AtEP3 expression level and the observed seed set defects is not very likely.

Analysis of embryogenic capacity in the Pe35S::AtEP3 and Pe35S::α-AtEP3 transformants

Since a role of the EP3 endochitinase was so far only demonstrated in carrot somatic embryo development, we continued our analysis by looking at the formation of somatic embryos in several of the transformants we generated. In *Arabidopsis*, embryogenic cultures can be established by direct seed germination in auxin-containing medium, which only works well for some mutants, or by using dissected immature zygotic embryos (Mordhorst *et al.*, 1998). We used the first method for the *Pe35S::AtEP3* lines and the second for the *Pe35S::α-AtEP3* The results are described in Table 4.

First of all, the success rate in obtaining embryogenic structures was rather low in all cases, and the qualitative aspect of these structures, even in the positive control *pt*, was not very good, probably due to suboptimal culture conditions. Some of the overexpression lines did give a few embryogenic structures after 3 weeks of culture whereas the wild-type did not. Some did look reasonably good and remained embryogenic for as long as 7 weeks (C6-8, D8-

1, D8-5, D8-6 and D19-9), but most of them gave no or poorly embryogenic structures and lost their embryogenicity with time. Although these were promising observations, when comparing the results with the expression levels found in the RT-PCR analysis (Figure 2B) we cannot correlate the increase in embryogenic capacity with a higher expression level of the *AtEP3* gene. For example, D8-4 and D8-5 have similar AtEP3 expression levels, comparable to the wild-type, yet D8-5 was one of the best cultures, whereas D8-4 did not give any embryogenic structure. The same is true for the D19 family, all of which have higher AtEP3 expression levels than the wild-type but only D19-9 produced an embryogenic culture. The C4-5 line was not tested because of the limited seed set in this line.

Line	Seedlings / embryos tested	After 3 weeks total ES	After 3 weeks Emb. cap.	Emb. cap. after 7 weeks
pt	37	5	+++	++
WS	122	0	NE	NE
C6-2	9	2	±	NE
C6-3	56	1	-	NE
C6-8	40	1	+	+
D8-1	76	5	±	±
D8-2	77	0	NE	NE
D8-3	77	0	NE	NE
D8-4	71	3	NE	NE
D8-5	77	2	+	+
D8-6	91	5	+	+
D19-1	43	0	NE	NE
D19-2	89	0	NE	NE
D19-5	32	1	NE	NE
D19-6	71	0	NE	NE
D19-9	94	1	±	+
WS	s	nd	±	±
B1-2	nd	nd	±-	±-
B3-3	nd	nd	±-	-

Table 4. Analysis of embryogenic capacity in the *Pe35S::AtEP3* and *Pe35S::α-AtEP3* lines.
This table shows examples of T_2 lines tested for their ability to form somatic embryos via direct seedling germination (*Pe35S::AtEP3* lines descending from T_1 lines C6, D8 and D19) or after immature zygotic embryo dissection (last three examples; *Pe35S::α-AtEP3* lines descending from T_1 lines B1 and B3). The formation of embryogenic structures (ES) was scored after 3 weeks of culture as well as their embryogenic capacity (Emb. cap.). This appreciation was mostly based on the color and the smoothness of these structures and was noted on a scale of - or + signs, going from - to +++. The greening of calli was directly correlated with embryogenicity. This observation was repeated weekly and after 7 weeks, cultures were considered embryogenic if there were still greenish and smooth ES were present. The *primordia timing (pt)* mutant was used as a positive control for the establishment of embryogenic lines via direct seedling germination (Mordhorst *et al.*, 1998). Non-embryogenic structures are noted NE.

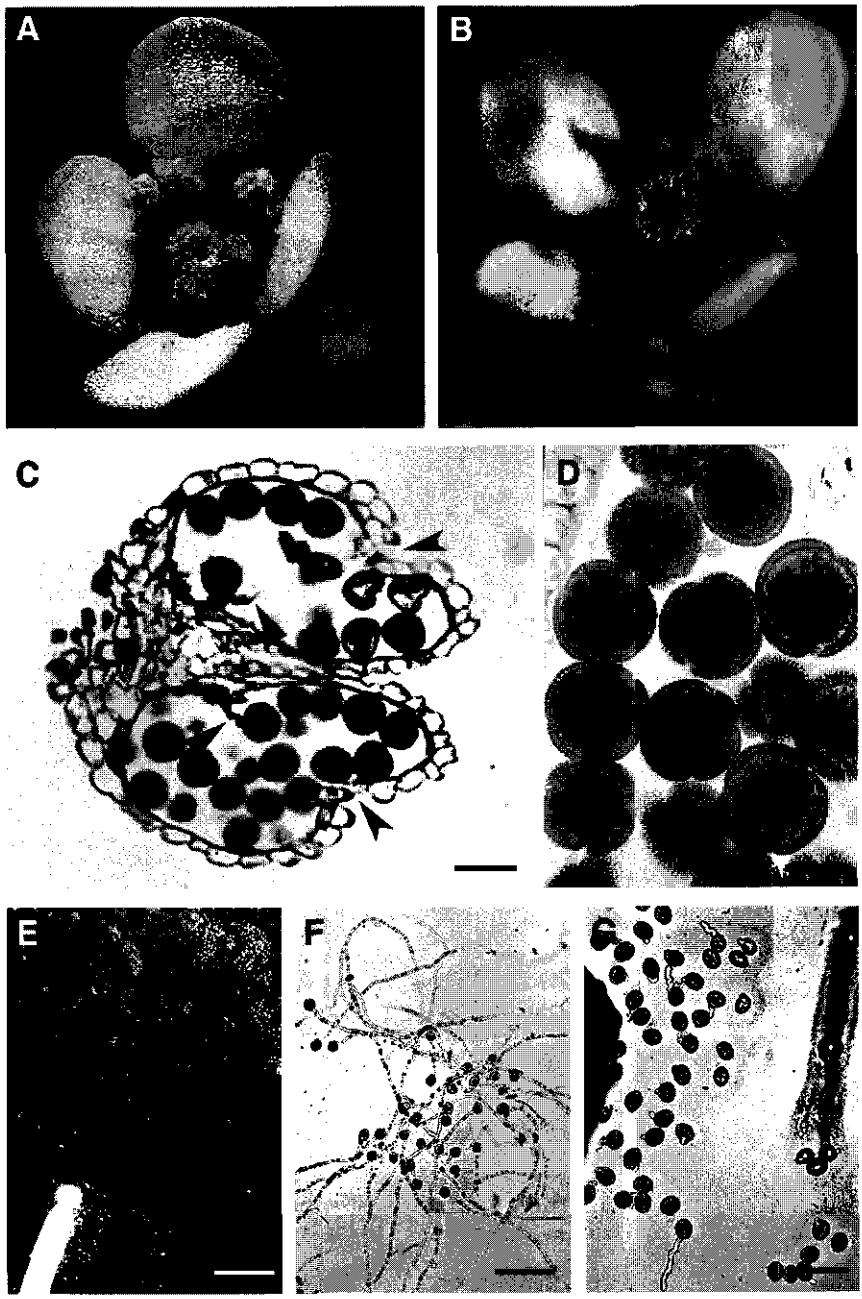
A similar conclusion could be drawn for the two antisense lines we tested. Embryogenic structures did form in both to the level of the wild-type, which was already very low. The two lines maintained their embryogenic capacity although it degraded with time for line B3-3. Considering the poor quality of the wild-type cultures as well, it was difficult to estimate whether an effect of the transgene was visible here.

Analysis of the sterility phenotype in the Pe35S::α-AtEP3 transformants

Regardless of the possible lack of correlation with the *AtEP3* gene, the sterility phenotype observed in the B3 antisense line was interesting enough by itself to pursue its analysis further. Since none of the overexpression lines displayed a similar phenotype they were not included in this analysis.

Flowers from sterile B3 plants carried no pollen on the anthers nor on the surface of the stigma (Figure 3A and B). Manual opening of the anthers revealed that pollen was produced but was apparently not released from the locules. This was confirmed by sectioning of these flowers (Figure 3C) and showed that dehiscence was not completed. The septum seems to undergo normal degradation (small arrows in Figure 3C) but the stomium on the other hand appears intact (arrowheads in Figure 3C) and as a result the locules are unable to open and release the pollen. A closer look at the pollen grains does not reveal any morphological defect (Figure 3D). We analyzed the viability of the pollen grains by their ability to germinate and form pollen tubes in vitro, according to Krishnakumar and Oppenheimer (1999). Figure 3E shows the experimental set up, i.e. the stigma of a wild-type flower laid on the surface of solid germination medium surrounded by pollen grains.

Figure 3. The male sterility phenotype of *AtEP3* antisense line B3.
A. Close view of a WS wild-type flower post-anthesis, on which pollen is clearly visible on the outside of the opened anthers and at the surface of the stigma. **B.** On a B3 sterile flower at the same stage as the wild-type flower in A no pollen is visible, neither on the anthers nor on the stigma. **C.** Cross section of an anther from a B3 sterile flower. Pollen grains are visible within the locules and dehiscence was initiated since the septum has degraded with only some tissue left (small arrows), whereas the stomium is still intact (arrowheads) prohibiting pollen release. Bar = 28 μm. **D.** High magnification of pollen grains within a locule of a B3 sterile flower. Bar = 10 μm. **E.** Overview of the experimental setup used for pollen in vitro germination, with a stigma laid on the surface of the solid germination medium and pollen grains dispersed on the same medium covered by a drop of liquid germination medium (Krishnakumar and Oppenheimer, 1999). The pollen grains on this view are from the WS wild-type and have already germinated with elongated pollen tubes clearly visible. Bar = 10 μm. **F.** Example of pollen germination in the wild-type. Nearly all pollen grains have germinated. Bar = 150 μm. **G.** Example of pollen germination with pollen from B3 sterile flowers. Only one pollen grain was considered as having germinated in this microscope field, at the bottom. Germination was initiated in most cases but pollen tubes did not elongate. Bar = 75 μm.


A
B
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G

The results visible in Figure 3F and G are summarized in Table 5. The percentages of germination obtained clearly show that the pollen of homozygous B3 sterile plants poorly germinates with values only around 3%, whereas in heterozygous non-sterile plants of the same line a pollen germination at the wild-type level is observed. Since the pollen of sterile flowers had to be released manually from the anthers we cannot exclude that the poor germination frequencies could partly be the result of physical damage. Moreover, as a result of the manual release less pollen grains could be analyzed.

Line	Germination [a]	Total [b]	% germination [c]
B1-16-2	144	229	62.9
B1-17-2	150	258	58.1
B2-4GG-1	105	208	50.5
B2-10	77	249	30.9
B2-15	96	204	47.1
B3-6-1 het.	169	279	60.6
B3-6-1 hom.	2	72	2.8
B3-8-1 hom.	3	107	2.8
WS	160	243	65.8

Table 5. Analysis of pollen in vitro germination on the *Pe35S::α-AtEP3* lines.
[a] number of germinated pollen grains with a pollen tube length at least twice the diameter of the pollen grain. [b] total number of pollen grains counted. [c] number of germinated pollen grains ÷ total number of pollen grains x 100. A number of homozygous descendants of T_1 lines B1 and B2 are shown here as well as descendants of T_1 line B3. 'het.' is for heterozygous and 'hom.' is for homozygous.

Homozygous descendants of line B2 do show somewhat reduced pollen germination compared with the wild-type, which could reflect the reduced percentages of seed abortion that were found (see Table 3). However, line B1 that shows a similar frequency of seed abortion does not seem to have a pollen germination defect.

To verify whether the sterility observed was only due to a defect in the male gametophyte we performed cross-pollinations using wild-type pollen. Normal silique elongation was observed, suggesting that ovule development is not affected. Sectioning of sterile flowers before fertilization also showed that ovule morphology was normal (Figure 4A) and that after fertilization the embryo sac undergoes degeneration (Figure 4B), which corresponds to the early aborted seeds we found (see Table 3). We tried to pollinate wild-type plants with pollen manually released from anthers of sterile plants but none of our crosses succeeded. We did not investigate whether the amount of pollen released was too low for successful pollination, whether it was damaged by the release procedure or whether it was caused by a mutation due

to the transgene. The escaped seeds we found on sterile plants and the in vitro germination tests seem to indicate that the pollen is still viable, although in lower amounts.

In conclusion, the strong sterility phenotype observed in line B3 could be the result of a double defect, i.e. no dehiscence and therefore no pollen release and reduced pollen germination rate affecting fertilization even further.

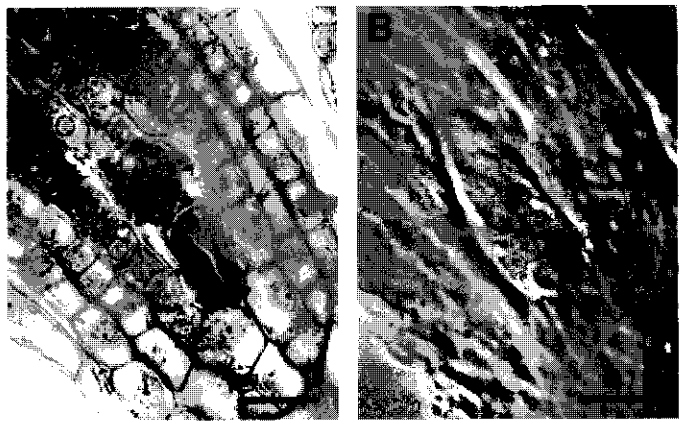

Figure 4. The female gametophyte in sterile flowers of *AtEP3* antisense line B3.
A. Longitudinal section of an ovule before fertilization from a flower bud of a B3 sterile plant. All cells of the embryo sac are present and look normal. **B.** Cleared ovule from an opened B3 sterile flower for which fertilization could not occur. Cells of the embryo sac are no longer distinguishable from one another as if cell boundaries had disappeared and only some nuclei remain visible, showing that the embryo sac cells have degenerated in the absence of fertilization. Bar = 180µm.

Analysis of the transgene insertion site in the antisense line B3

The absence of *AtEP3* mRNA quantification in the antisense lines led to us to analyze the insertion site of the *Pe35S::α-AtEP3* transgene in the male sterile line B3. We first determined the number of transgene copies inserted per genome by genomic Southern blotting using a fragment of the NPTII gene as a probe (Figure 1B). This was done together with the B1 and B2 lines. Figure 5A shows an example of hybridization pattern obtained with plants from two generations. The autoradiogram clearly shows that some of the transgenic plants have multiple copy insertions and that these insertions are segregating within the families. This also confirms the results of the kanamycin selection (Table 2). Plants B1-13 and B1-15, for example, have an identical hybridization pattern that is probably very

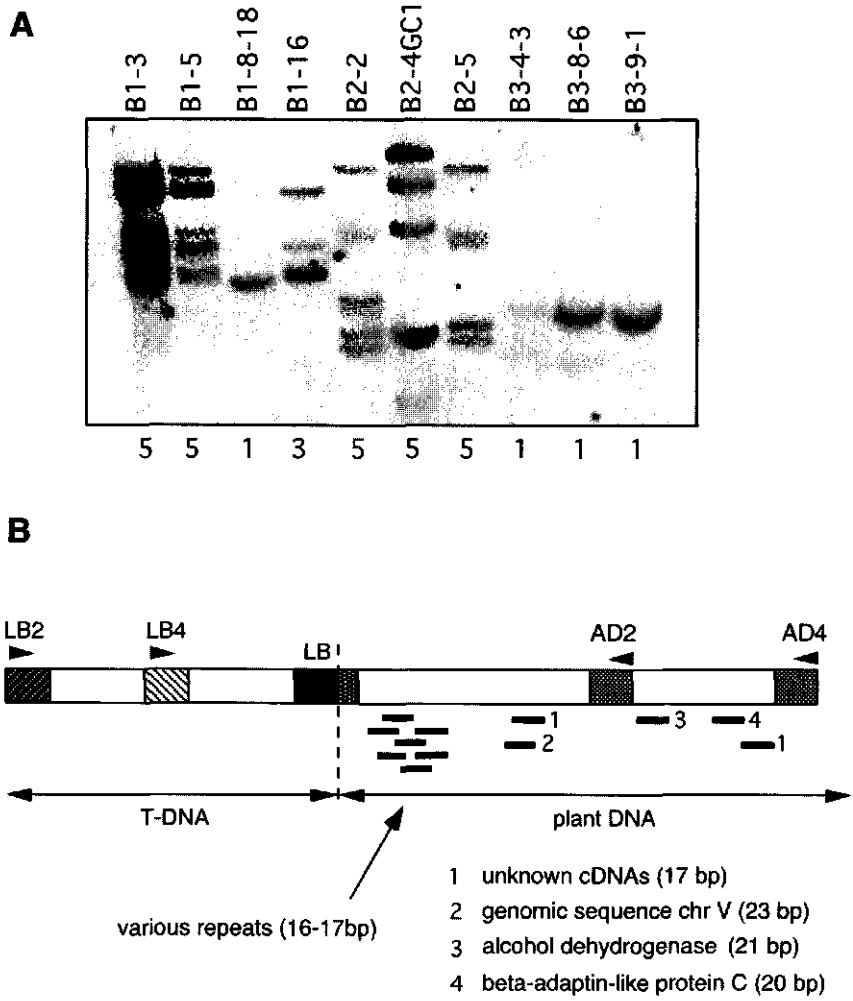

Figure 5. Analysis of the T-DNA insertion site in *AtEP3* antisense line B3.
A. Autoradiogram of a Southern blot performed on genomic DNA from *Pe35S::α-AtEP3* transformants restricted with *EcoRI* and hybridized with the probe indicated in Figure 1B. The name of the samples is indicated above the lanes. Some descendants of the three T_1 lines, B1, B2 and B3 are represented and the nomenclature used is as in Figure 2, i.e. B3-8-6 is the 6^{th} T_3 line descending from the 8^{th} T_2 line descending from T_1 line B3. The numbers under each lane represent the estimated number of T-DNA insertions based on the hybridization pattern. **B.** The area amplified by TAIL-PCR and sequenced is schematically represented, with the position of the primers used. LB2 was used in combination with AD2 or AD4 for the second PCR round and LB4 was used instead of LB2 in the third PCR round. The four PCR products generated by the four primer combinations were sequenced and assembled in the DNA fragment shown here, going from primer LB2 to primer AD4. The DNA sequences belonging either to the T-DNA construct or to the plant genome are indicated, as well as the stretches of plant DNA found homologous to known sequences identified by the numbers.

similar to the one of the mother plant B1. On the other hand, plant B1-16, descending from the same plant, only has three common bands left and in the case of B1-8-18, that is already one generation further, only one band remains. This segregation is also clearly visible for descendants of transformant B2. The B3 family is the only antisense line with a single transgene insertion, as suggested by the results of the kanamycin selection (Table 2). Based on this result we could confidently undertake the isolation of the DNA flanking the transgene insertion site.

For this purpose we employed TAIL-PCR (Liu *et al.*, 1995) and were successful in generating a number of DNA fragments with two of the degenerate primers (AD2 and AD4). All PCR products originated from the left border of the T-DNA, suggesting that, as a result of the insertion, the right border was probably not intact anymore. The amplified DNA fragments were subsequently analyzed by sequencing. This revealed that they were overlapping and all contained the left border of the T-DNA. Figure 5B shows a schematic representation of the sequenced area. BLAST searches were performed on the longest sequence and only very small stretches between 16 and 23 nucleotides were found to be homologous to known sequences, including several repeats. No significant homology was found suggesting that the transgene was not inserted in a coding sequence or in a sequenced area of the *Arabidopsis* genome, since no BAC sequence was identified by the BLAST search. The latter fact and the presence of several repeats in this DNA fragment suggest that the T-DNA might have landed in a heterochromatic region. This indicates that in the B3 antisense line no recognizable coding sequence is disrupted by the transgene. However, additional sequence information, especially from the right border of the insertion site is needed to make a more definite statement. Unfortunately, no flanking DNA could be amplified from this end of the T-DNA.

Morphological analysis of root hair formation in the Pe35S::AtEP3 and Pe35S::a-AtEP3 transformants

The *AtEP3* gene is also expressed in elongating root hairs and it was therefore a logical next step to look at root development in the lines we generated. There were no differences found in most of the lines we analyzed. Root length seemed normal and there was no obvious defect in root and root hair morphology. However and interestingly, line B3 did show a root hair phenotype. The number of root hairs produced was indeed dramatically reduced, as can be seen in Figure 6A-C, especially in homozygous seedlings (Figure 6C).

We found another root phenotype in line C4-5, in which root hair and/or epidermis cell morphology seemed affected. Cells appeared swollen and deformed (Figure 6D).

In conclusion, from the phenotypic and molecular analysis of both AtEP3 overexpression and antisense lines generated, only one line was recovered in which three phenotypes were correlated with a reduction in AtEP3 expression to 10% that of the wild-type. This line, C4-5, exhibits a root epidermal cell defect, defective transmission and a high level of seed abortion. While the latter two phenotypes were also found in lines with normal *AtEP3* mRNA levels, the defect observed in root epidermal cells may be the most specific effect of a reduction in *AtEP3* mRNA level. More detailed analyses still need to be performed. This is also true for the B3 antisense line that shows a male sterility phenotype and reduced root hair growth, but for which no link with the *AtEP3* gene could be established.

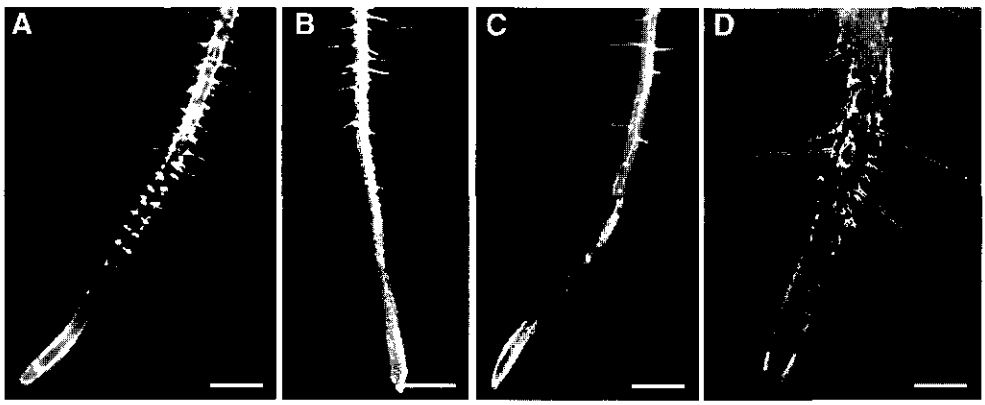

Figure 6. Analysis of root hair growth in *AtEP3* antisense and overexpression lines.
A. WS wild-type seedling root showing normal root hair growth. **B.** Heterozygous antisense B3 seedling root, showing a longer elongation zone and fewer root hairs than the wild-type. **C.** Homozygous antisense B3 seedling root, showing an even longer elongation zone than in heterozygous seedlings and hardly any root hairs. **D.** Root of a seedling from co-suppression line C4-5. Root hairs cells appear swollen. In A and C, Bar = 0.8 mm, in B, Bar = 1 mm and in D, Bar = 0.5 mm.

Discussion

In this work, we have addressed the role of the AtEP3 endochitinase during *Arabidopsis* development by means of a reverse genetics approach. Our aim was to generate transgenic plants with an altered expression of the chitinase gene by using an overexpression and an antisense construct. A number of transformants were successfully generated for both

constructs and molecular analysis showed that the *AtEP3* mRNA levels were successfully affected by the presence of the overexpression construct with several lines showing increases up to 7-fold as compared to the wild-type, and a line showing a 10-fold decrease. However, due to technical difficulties the *AtEP3* mRNA level could not be measured in the antisense lines.

DNA and genetic analysis showed that the transgenes had been inserted in multiple copies in a number of transgenic lines. We did not determine their mode of insertion, i.e. tandem repeats, inverted repeats, or isolated copies since in these plants the transgene was often silenced in the subsequent generation as indicated by a loss of kanamycin resistance. This phenomenon is frequently observed in the analysis of transgenes using this type of approaches (for a review see Kooter *et al.*, 1999). Analysis of the transgene segregation also revealed transmission defects, indicative of embryo and/or gametophytic lethality, but they were not correlated with a change in *AtEP3* mRNA level.

Based on the expression pattern of the *AtEP3* gene (Passarinho *et al.*, 2001), we performed a phenotypic analysis focused on seed set, pollen development, root hair formation and somatic embryo development. In general, increasing the AtEP3 expression level up to 7-fold did not seem to have any visible consequence on any of these processes. We only observed differences with the wild-type in the case of line C4-5. Since AtEP3 expression could not be quantified in antisense lines, it was not possible to correlate the phenotypes observed in the latter and therefore could not be compared with those of line C4-5.

Antisense line B3 did show two interesting phenotypes that appeared genetically linked with the presence of the transgene. B3 plants homozygous for the transgene are male sterile and are therefore not able to produce seeds, apart from some rare escapes. We established that this recessive phenotype is due to incomplete dehiscence. The septum seems to degenerate but the stomium remains intact and although pollen is produced it cannot be released. The female gametophyte is not affected as demonstrated by microscopic observation and pollination with wild-type pollen. It also appeared that when released manually B3 homozygous pollen germinates poorly, but is probably still partly viable as suggested by the few seeds that were produced in these plants. The number of root hairs was also dramatically reduced as compared to the wild-type. These observations could have matched a defect in AtEP3 expression, since the gene was shown to be expressed in maturating pollen, growing pollen tubes and root hairs (Passarinho *et al.*, 2001). However, in the absence of *AtEP3* mRNA quantification it could also be that these phenotypes are the result of a gene disruption by the T-DNA construct. The insertion site of the antisense construct was analyzed by TAIL-

PCR (Liu *et al.*, 1995) to verify this possibility. Sequencing revealed that the *Pe35S::α-AtEP3* construct was not inserted in any coding sequence. The very small stretches of homology found in the analysis were not consistent with a precise chromosomal location and together with the numerous repetitive sequences identified it could suggest that the T-DNA was inserted in a heterochromatic region of the *Arabidopsis* genome that remains unsequenced. Unfortunately, this sequence analysis could not be performed on DNA flanking the T-DNA right border, most likely due to a rearrangement of the latter while inserting in the plant DNA. Therefore we cannot draw definite conclusions from the analysis of the insertion site. It could also be that the transgene inserted into some essential regulatory sequence present in heterochromatin and would act in trans on a gene involved in pollen and root hair development. There are only few examples of similar male sterility phenotypes and none of the corresponding genes has been isolated so far (Park *et al.*, 1996; Sanders *et al.*, 1999). Based on their chromosomal locations, these mutations represent different genes, none of which can be *AtEP3*. It has also not been reported whether root hair formation is also altered in these mutants. The only described mutant that is affected in both pollen and root hair development is *tip1* (Schiefelbein *et al.*, 1993). T*ip1* homozygous plants are not fully sterile and although the mutated gene, that remains unidentified, was proposed to function in tip growth it is not likely to be the one affected in our B3 plants. If an essential pollen-specific gene was defective in these plants, a large majority of the pollen grains would be unable to complete maturation. As a result germination would only occur in small numbers, as we indeed observed in our in vitro germination tests. This could also indirectly influence a proper opening of the anthers if signals were to be sent from the mature pollen to the locule in order to trigger a cascade of events leading to dehiscence.

The only line for which we could correlate a phenotype with a change in AtEP3 expression did not show any defect in anther dehiscence. Pollen is indeed released normally in line C4-5 that has a 10-fold decrease in AtEP3 expression. Although the 1:1 segregation ratio of the transgene together with the observed reduced seed set suggest gametophytic lethality we do not think that the latter phenotypes are linked with the *AtEP3* gene. There was indeed too much variation between two generations. Furthermore, no correlation could be made with the AtEP3 expression levels. Similar seed set and transmission defects were also observed in lines that had similar or higher levels of *AtEP3* mRNA than the wild-type. Root hair formation is affected in line C4-5 only and this is in agreement with the expression of the AtEP3 in root hairs (Passarinho *et al.*, 2001). Root hairs do form and grow but they are swollen, suggesting a weakened cell wall. Interestingly, (Ding and Zhu, 1997) showed that blocking AGP activity

by using β-glucosyl Yariv reagent resulted in a very similar phenotype, while the same phenotype was also observed in the *reb1-1* mutant (Baskin *et al.*, 1992). It was also noted in this study that the AGP profile in the *reb1-1* mutant is different than in the wild-type, suggesting that specific AGPs are absent or are not processed properly. This could also be in agreement with a defect in AtEP3 expression in relation with cell wall AGPs and lends support to a role of the AtEP3 chitinase in the processing of signal molecules.

In conclusion, this work shows that down-regulation of the *AtEP3* gene at the RNA level can affect root hair morphology and is therefore most likely involved in a developmental process.

Materials and Methods

Plant material

Arabidopsis thaliana (L.) Heynh plants were grown under long day light conditions after germination on 0.5x MS salts (Murashige and Skoog, 1962), 1% (w/v) sucrose, 0.8% (w/v) agar. The ecotype *Wassilewskija* (WS) was used as wild-type and for plant transformation. Transformed plants were selected on the same germination medium containing 100 μg ml^{-1} kanamycin sulphate (Duchefa, Harleem, The Netherlands).

Construction of AtEP3 overexpression and antisense cassettes and plant transformation

The *AtEP3* cDNA was cloned between the enhanced CaMV 35S promoter (Pe35S) and the NOS terminator (NOS3) in the vector pMON999 (van Bokhoven *et al.*, 1993) as a *Bgl*II-*Kpn*I fragment for the overexpression construct and as a *Kpn*I-*Bgl*II fragment for the antisense construct. The *Kpn*I and *Bgl*II sites were engineered at the extremities of the cDNA by PCR mutagenesis, using the primers 5'taiBglII (5'-GAAGATCTTCAAAGTG CTCCCACC-3') and 3'taiKpnI (5'-CGGGGTACCCCGAAATAGATGTTTTTGTTAGC-3') or 5'taiKpnI (5'-GGGGTACCCCTATTCCTCAACAACATC-3') and 3'taiBglII (5'-GAAGATCTTTAGATGTTTTTGTTGCAAGTGAGG-3') for the overexpression and the antisense construct, respectively. The Pe35S::AtEP3::NOS3 and the Pe35S::α-AtEP3::NOS3 cassettes were subsequently introduced into the binary vector pMOG800 (Figure 1A-B; kindly provided by Mogen-Zeneca, Leiden, The Netherlands) and the resulting constructs were transformed into *Arabidopsis* plants, ecotype WS, by vacuum infiltration according to Bechtold *et al.* (1993). Twelve plants were transformed per construct and named from A to L. Transformants were then selected on kanamycin at each generation and named after their mother plant, e.g. T_1 transformants derived from plant A, were named A1 to Ax, T_2

descendants were named A1-1 to Ax-x, T_3 descendants were named A1-1-1 to Ax-x-x, and so forth.

DNA analysis

Genomic DNA was isolated from individual kanamycin resistant plants of each transgenic line as well as from wild-type plants, according to Kozik *et al.* (1996). The presence of the transgene in the genome of the transgenic plants was confirmed molecularly, by PCR analysis using construct-specific primers (Figure 1A-B: 35S-5', 5'-TCTGTCACTTCATCAAAAGG-3', and TAI2, 5'-TGTTAGCAAGTGAGGTTGTTTC CAGGATCA-3' (for the overexpression construct), or PP5, 5'-ATATCCTTCAAAA CCGCCTTGTGGTACTGG-3' (for the antisense construct)). The number of copies inserted per genome was determined by genomic Southern blotting as described in Passarinho *et al.* (2001) using the enzymes *EcoR*I and *Kpn*I and a radiolabeled fragment of the neomycin phosphotransferase II gene (NPTII, Figure 1B) as a probe.

RT-PCR analysis

Total RNA from seedling and inflorescences was isolated according to Kay *et al.* (1987). Total RNA was also isolated from pollen grains using TRIZOL® reagent (Invitrogen Life Technologies, Breda, The Netherlands) according to manufacturer's recommendations. Pollen grains were isolated from about 100 flowers essentially according to Treacy *et al.* (1997).

RNA was DNAse-treated for 30 min at 37°C with 3 units RQ1 DNAse (Invitrogen Life Technologies) in 40 mM Tris-HCl pH 8.0, 10 mM NaCl, 6 mM $MgCl_2$ and 10 mM $CaCl_2$. After phenol/chloroform extraction and ethanol precipitation, the RNA was resuspended to a concentration of 500 ng μl^{-1}. Two µg DNAse-treated RNA were reverse-transcribed for 1h at 37°C, using 1 µg oligo-dT_{12}, 1mM dNTPs, 40 units RNAse out (Invitrogen Life Technologies) and 200 units M-MLV reverse-transcriptase (Invitrogen Life Technologies) in 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$ and 5 mM DTT. Presence of remaining genomic DNA was verified by a control reaction performed for each sample without reverse-transcriptase. After enzyme denaturation for 5 min at 95°C and a 20-fold dilution, 2 µl reverse-transcribed RNA were analyzed by PCR, using *AtEP3* gene-specific primers (PP1, 5'-TTCGTCAGAGCTATGTTGTAGTCAGTTTGG-3', and PP6, 5'-CCACAAGGCGGTTTT AGATATGACTGG-3' or AtEP3-3'rev, 5'-CCATTCCATTCTTAAAGCTTGTCTATT-3') and, as an internal standard for quantification, primers specific for the constitutive cyclophilin

gene *ROC5* (Chou and Gasser, 1997; ROC5-5', 5'-TCTCTCTTCCAAATCTCC-3', and ROC5-3', 5'-AAGTCTCTCACTTTCTCACT-3'). PCR products were analyzed by agarose gel electrophoresis, Southern blotting and hybridization to radiolabeled gene-specific probes. Quantification of the autoradiogram signals was carried out using the software ImageQuant® (Molecular Dynamics, Sunnyvale, CA, USA).

Phenotypic analysis

Pollen in vitro germination tests were carried out as described by Krishnakumar and Oppenheimer (1999). Germination was scored per microscope field by counting the number of pollen tubes with a length at least twice the diameter of the pollen grain and the total number of pollen grains. The germination frequencies were determined on the added numbers of all microscope fields.

Seed set was analyzed by collecting about 10 siliques per plant starting from the fourth silique below the terminal inflorescence. Siliques were opened under a Nikon SMZ-2T binocular microscope and seeds were counted and classified according to their stage of development: early aborted (white and tiny), late aborted (brown and shrunken) and fully developed. Percentages of seed abortion per silique and per plant (total early plus late) were then determined.

Embryogenic capacity of the transgenic lines was analyzed in vitro by establishment of embryogenic cultures as described by Mordhorst *et al.* (1998). Overexpression lines were tested in conditions where *Arabidopsis* wild-type is not able to form somatic embryos, i.e. directly from seedlings (Mordhorst *et al.*, 1998). Antisense lines, on the other hand, were tested by using dissected immature zygotic embryos, a method that allows the formation of somatic embryos in wild-type (Mordhorst *et al.*, 1998). Embryogenic capacity was scored the third week after initiation by counting the number of embryogenic clusters formed versus the number of seeds or embryos used to initiate the culture. The evolution of these clusters was followed weekly and compared to the wild-type cultures.

Cytological observations were performed on sections of flowers after fixation in 3.7% (v/v) formaldehyde, 50% (v/v) ethanol and 5% (v/v) acetic acid, followed by embedding in Technovit 7100 (Heraeus Kulzer, Wehrheim, Germany) according to the manufacturer's recommendations. Embedded material was sectioned with a microtome to a thickness of 3 µm and was subsequently stained in 1% (w/v) toluidine blue and 1% (w/v) sodium tetraborate. Sections were observed after mounting in Euparal (Agar Scientific, Stansted, UK). Observations of ovules were also performed with Nomarski optics after clearing of opened

carpels in a solution of 8 g chloral hydrate (Merck, Darmstadt, Germany) in 2 ml water and 1 ml glycerol.

TAIL-PCR

TAIL-PCR was performed on genomic DNA from B3 plants essentially as described by Liu *et al.* (1995), replacing the Ds primers by nested primers specific for the T-DNA right and left border. The primers were designed to allow band shifts detectable on a 3% (w/v) agarose gel. Primers for the right border were, in outwards direction: RB1, 5'-ACAACGTCGTGACTGGGAAAACC-3'; RB2, 5'-AGCTGGCGTAATAGCGAAGAGG-3' and RB3, 5'-ATCAGATTGTCGTTTCCCGCC-3'. Primers for the left border were, in outwards direction: LB1, 5'-AGCGGGACTCTGGGGTTCG-3'; LB2, 5'-AATGTGTGAG ATCAAGG-3' and LB4, 5'-TCCTAAAACCAAAATCCAGTACTAAAATCC-3'. PCR products were analyzed by agarose gel electrophoresis and bands giving the expected band-shifts were purified from gel using an Agarose Gel DNA Extraction Kit (Roche Molecular Biochemicals, Mannheim, Germany), cloned in the pGEM-T vector (PROMEGA, Madison WI, USA) and sequenced.

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Chapter 6

The chitinase mutant *ep3-1* is affected in seed germination, root hair and pollen development

Abstract. The *Arabidopsis AtEP3* gene was isolated based on its homology to the carrot EP3 endochitinase gene. Previous work has shown that the *AtEP3* gene is expressed in maturing and germinating pollen, in growing pollen tubes, in the endosperm cap or inner layers of the seed coat upon germination and in elongating root hairs. Expression was also found in leaf hydathodes and stipules and in embryogenic suspension cultures. In this work, we present a mutant transgenic line with an insertion into the second exon of the *AtEP3* gene. As a result of the insertion no *AtEP3* mRNA is produced and the corresponding plants display a range of subtle phenotypes that, eventhough further investigations are required, are in agreement with the expression pattern of the gene.

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Introduction

Since it was first demonstrated that a bean endochitinase (EC 3.2.1.14) could have an antifungal activity (Schlumbaum *et al.*, 1986), plant chitinases have received great attention for their potential use in pest control in agriculture. Numerous studies were therefore conducted with the preconceived idea that the natural role of plant chitinases is defense against fungal pathogens. In vitro experiments in the line of Schlumbaum's work in other plant species and on other chitinases confirmed this idea (Arlorio *et al.*, 1992; Garcia-Casado *et al.*, 1998; Mauch *et al.*, 1988). Besides, a number of findings also strengthened this opinion by showing that transgenic plants with increased levels of chitinase were less sensitive to certain fungi (Broglie *et al.*, 1991; Grison *et al.*, 1996; Jach *et al.*, 1995). However, in other studies similar approaches failed to demonstrate such an effect (Neuhaus *et al.*, 1996; Samac and Shah, 1994). Furthermore, other studies indicated that the expression of some chitinases was developmentally regulated, with specific isoforms appearing in certain organs only during certain stages of development (Neale *et al.*, 1990; Trudel *et al.*, 1989). And there is now growing evidence that at least some of these chitinases might actively participate to developmental processes such as embryo development (de Jong *et al.*, 1992; van Hengel *et al.*, 2001) and pollination (Leung, 1992). The role of chitinases appears therefore more general and there might be many functions, in line with the large families of chitinase genes present (reviewed in Graham and Sticklen, 1994). This makes the mutational analysis of a single particular chitinase rather complicated, since not only a broad spectrum of processes needs to be tested, but also problems generally attributed to genetic redundancy may arise.

In this work we have addressed the role of the *Arabidopsis thaliana* (L.) Heynh AtEP3 chitinase (Passarinho *et al.*, 2001), the ortholog of the carrot EP3 endochitinase, which was shown to play a crucial role during somatic embryo development (de Jong *et al.*, 1992). The EP3 chitinase is able to lift the arrest imposed on somatic embryos of the temperature sensitive carrot cell line *ts11* when grown at non-permissive temperature (de Jong *et al.*, 1992). Further work has shown that the EP3 chitinase is most likely acting via signal molecules containing N-acetyl glucosamine (GlcNAc) generated from a larger precursor (de Jong *et al.*, 1993). Recently it was found that certain arabinogalactan proteins (AGPs) are a potential substrate for the EP3 chitinase, since they not only contain cleavage sites for this enzyme but also the promoting effect of AGPs on somatic embryogenesis was enhanced after chitinase treatment (van Hengel *et al.*, 2001). AGPs are also often associated with developmental processes in several plant compartments (Knox, 1999), including embryos. In

combination with co-localizing specific chitinases, such as EP3, modulation of their activity through hydrolytic processing could be an important mechanism in plant development.

We have looked for knockout mutants of the *AtEP3* chitinase gene and have identified one insertion line among the Sainsbury Laboratory Arabidopsis Transposants (SLAT lines Sainsbury Laboratory, Norwich, UK). We have demonstrated that this line contains a *dSpm* insertion in the second exon of the *AtEP3* gene, which in homozygous plants results in a complete loss of *AtEP3* mRNA. We performed a detailed phenotypic analysis aided by the prior knowledge of the expression pattern of the *AtEP3* gene (Passarinho *et al.*, 2001) and have detected a range of subtle developmental phenotypes. Although these need to be further confirmed they are in general agreement with the expression pattern of AtEP3. To our knowledge, this is the first report of a functional analysis based on a chitinase knockout mutant.

Results

Identification of an AtEP3 insertion line

We have performed a BLAST search with the *AtEP3* gene sequence against sequenced inserts of *Arabidopsis* using the NASC SINS BLAST server (http://nasc.nott.ac.uk/blast.html). This server of the Nottingham Arabidopsis Stock Centre (NASC, Nottingham, UK) allows homology searches against Sequenced INsertion Sites (SINS) from several insertion collections. By this means we have identified in the SLAT collection of the Sainsbury Laboratory (Norwich, UK; Tissier *et al.*, 1999) 3 insertions into the AtEP3 coding sequence, all in the second exon (Figure 1). The corresponding pools (DNA and seeds) were obtained from the NASC and plants of each pool were grown and screened by PCR for the presence of a *dSpm* insertion in the *AtEP3* gene, using the pool DNA as a positive control. Two gene-specific primers were used in combination with two *dSpm* primers, each from a different extremity to cover all possible orientations. We could only identify a positive plant for pool 2_28 using the primers PP1 and dSpm11 (Figure 1A). The pools are made of seeds harvested from 50 different plants and we only grew 50 seeds of each pool. Therefore, there is a big chance that we missed the positive plant of the two other pools due to the small scale of our screening. However, we continued with the plant we had identified (2_28_5).

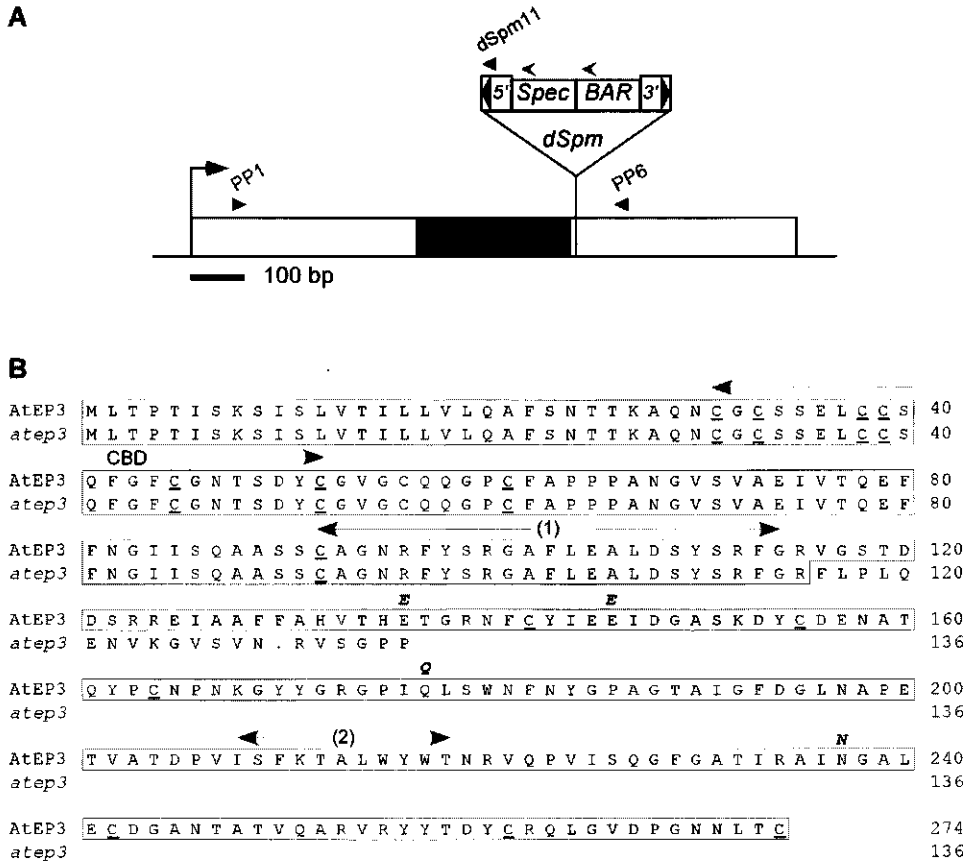

Figure 1. Disruption of the *AtEP3* gene by a *dSpm* insertion.
A. Schematic representation of the *dSpm* insertion site in the *AtEP3* gene. The coding sequence is represented by the open boxes surrounding the intron (shaded box). The primers used to confirm the insertion and for RT-PCR are represented by the arrowheads. Representation of the *dSpm* element is adapted from Tissier *et al.* (1999). **B.** Effect of the *dSpm* insertion on the AtEP3 protein sequence. The AtEP3 amino acid sequence (AtEP3) is here aligned to the translated sequence of the *AtEP3* gene after insertion of the dSpm element (*atep3*). The (.) stands for a stop codon and the arrows indicate the functional domains of the protein. CBD stands for chitin-binding domain, which is followed by the catalytic domain made of regions (1) and (2) containing consensus sequences for PROSITE signatures Chitinase 19_1 and Chitinase 19_2, respectively. Cysteine residues forming disulfide bonds are underlined and residues indicated in bold and italic above the alignment are essential for catalytic activity (Garcia-Casado *et al.*, 1998).

Molecular analysis of the ep3-1 mutant

The precise insertion site in the *AtEP3* gene was confirmed by nested PCR using the dSpm5 primer, upstream of dSpm11. Products of both PCRs were purified and sequenced. The sequence analysis confirmed the presence of the *dSpm* element in the second exon of the *AtEP3* gene (Figure 1A). The sequence resulting from the insertion was translated and aligned

with the intact AtEP3 protein sequence (Figure 1B). This revealed that the insertion causes a frame-shift within the catalytic domain of the chitinase after the R_{115} residue followed by a stop codon 14 residues further. Although the chitin-binding domain, as well as the first region of the catalytic domain including one of the conserved regions, proposed to be involved in the hydrolytic activity of the enzyme are intact, the second conserved region and all residues essential for hydrolytic activity are missing (Garcia-Casado *et al.*, 1998). Therefore, if the disrupted *AtEP3* mRNA was still produced and translated, the corresponding protein would no longer be acting as an active AtEP3 chitinase.

We also determined by PCR the genotype of the identified plant for the *dSpm* insertion into *AtEP3*. For this purpose we analyzed DNA of this plant and of 53 of its descendants with 2 gene specific primers (PP1 and PP6) and the dSpm11 primer in the same reaction (Figure 1A). In case of a segregating population we would have amplified the wild-type allele only (PP1-PP6 PCR product) in the wild-type plants (1/4), both the wild-type and the insertional alleles (PP1-PP6 and PP1-dSpm11 PCR products) in heterozygous plants (1/2) and the insertional allele only (PP1-dSpm11 PCR product) in plants homozygous for the insertion (1/4).

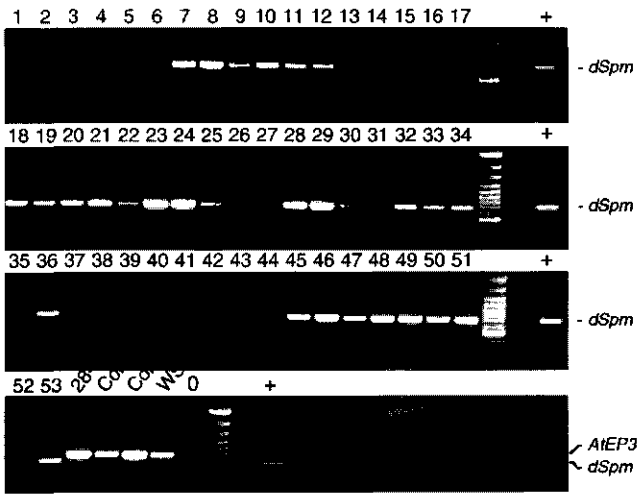

Figure 2. Genotype of SLAT line 2_28_5.
Agarose gel electrophoresis of the PCR products obtained by using the primers PP1, PP6 and dSpm11 on genomic DNA from plant 2_28_5 and 53 of its descendants (numbers above the lanes) as well as from plant 2_28_6, which originated from the same pool as 2_28_5 but does not have an insertion in *AtEP3*, plus from the wild-type ecotypes *Columbia* wild-type (Col-0) of two different DNA isolations and *Wassilewskija* (WS). The 0 indicates the negative control (no DNA). The sizes of the PCR product corresponding to the disrupted and intact *AtEP3* alleles are noted *dSpm* and *AtEP3*, respectively.

All plants were in the latter situation (Figure 2), indicating that the mother plant was already homozygous and that in all plants to be analyzed phenotypically both alleles of the *AtEP3* gene are disrupted.

Total RNA was isolated from flowers of 2_28_5 homozygous plants in order to verify the effect of the *dSpm* insertion on the level of *AtEP3* messenger. RT-PCR was performed with gene-specific primers for the *AtEP3* gene and the cyclophilin gene *ROC5* (Chou and Gasser, 1997), our internal standard. The results shown in Figure 3 clearly demonstrate that intact *AtEP3* mRNA is no longer produced in plants that are homozygous for a *dSpm* insertion into the AtEP3 gene. Therefore we have renamed the AtEP3 insertion line 2_28_5 as *ep3-1*.

Figure 3. No AtEP3 mRNA is produced in the *ep3-1* mutant.
Autoradiogram of a RT-PCR performed on flower RNA from *Columbia* wild-type (Col-0) and SLAT lines 2_28_5 (*ep3-1*) and 2_28_6 using the primers PP1 and PP6 (see Figure 1A). The upper panel shows the result obtained for the *AtEP3* gene, whereas the lower panel shows the results obtained for the internal standard we used, the cyclophilin gene *ROC5* (Chou and Gasser, 1997). As in Figure 2, plant 2_28_6 that does not have an insertion in *AtEP3* was used as an additional control.

Genetic and phenotypic analysis of the ep3-1 mutant

Based on the expression of the *AtEP3* gene in maturing and germinating pollen and also in growing pollen tubes (Passarinho *et al.*, 2001), we started our observations with pollen germination and performed a pollen in vitro germination test according to Krishnakumar and Oppenheimer (1999) with pollen from Col-0 and *ep3-1* homozygous plants. The results shown in Table 1 and in Figure 4 indicate that pollen germination is reduced in *ep3-1*. A minority of the pollen grains does germinate (Figure 4A and D) and some pollen tubes are able to elongate normally (Figure 4B-C and E-F) but the frequencies remain much lower than in the wild-type.

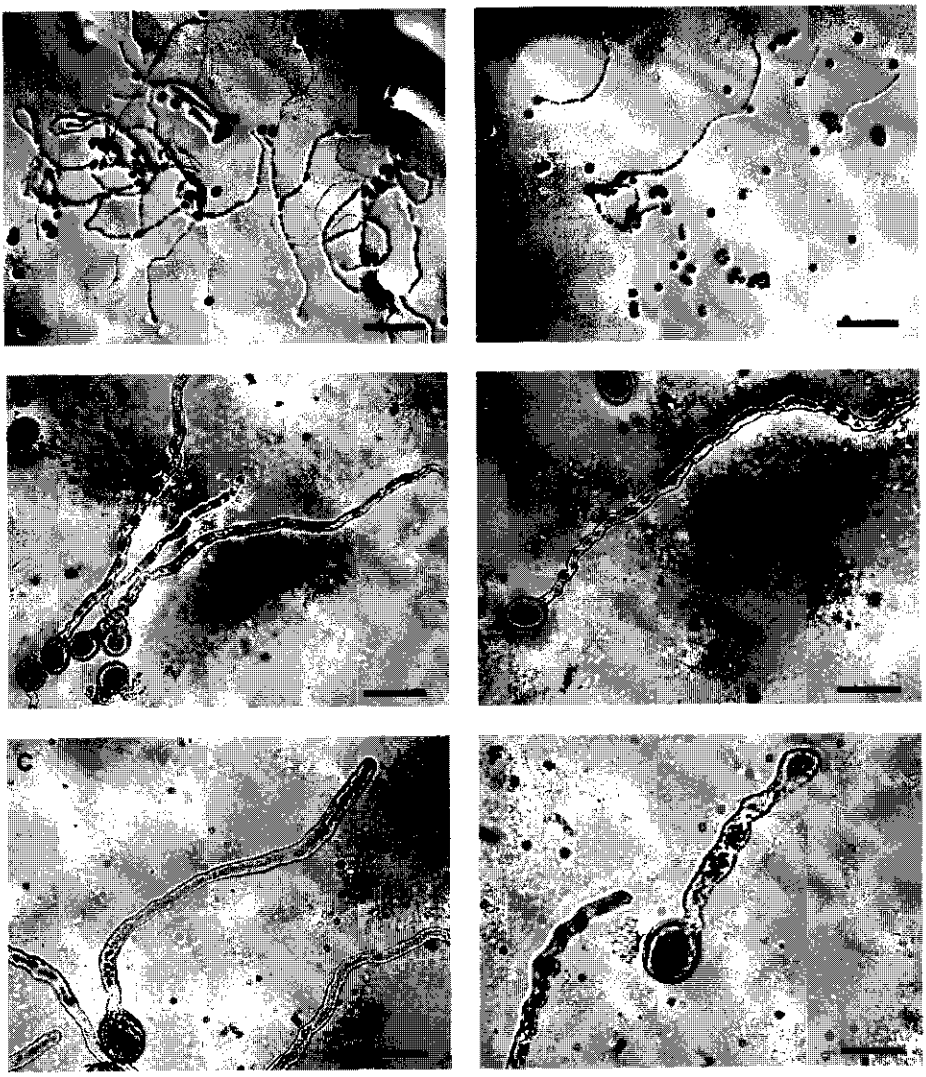

Figure 4. Pollen germinates poorly in the *ep3-1* mutant.
A-C. In vitro germination of *Columbia* wild-type pollen. **D-F.** In vitro germination of *ep3-1* pollen. Bar = 200 μm in A and D; 40μm in B; 20 μm in C; 35 μm in E and 25 μm in F.

We divided the surface of the germination medium in two zones to account for the influence of stigma exudates: (1) more than 5 millimeters away from the stigma placed on the surface of the medium and (2) closer than 5 millimeters. In the case of *ep3-1* pollen we could observe a large difference between the two zones (14% in (1) versus 54% in (2)) whereas in the case of wild-type pollen this difference was less obvious (68% in (1) versus 84% in (2)). This

suggests that stigma exudates are able to partly compensate for the poor germination rate of *ep3-1* pollen.

Line	Germination [a]	Total [b]	% germination [c]
ep3-1 [1]	158	1142	14
[2]	197	368	54
[3]	355	1313	27
Col-0 [1]	96	142	68
[2]	46	55	84
[3]	142	194	73

Table 1. Analysis of pollen in vitro germination in the *ep3-1* mutant.
[a] number of germinated pollen grains with a pollen tube length at least twice the diameter of the pollen grain. [b] total number of pollen grains counted. [c] number of germinated pollen grains ÷ total number of pollen grains x 100. [1] total of pollen grains counted in all microscope fields excluding the area around the stigma. [2] total of pollen grains counted in microscope fields around the stigma only. [3] total of pollen grains counted in all microscope fields.

The fact that some of the pollen can still germinate in the presence of stigma exudates is probably sufficient to allow normal fertilization in planta, since we observed no defects in seed set as compared to the wild-type. We only found one early aborted seed out of 415 in the siliques we looked at (8 out of 230 for Col-0). No further defect was observed in the developing seeds either. Previously we have shown that this chitinase is exclusively expressed in embryogenic cultures (Passarinho *et al.*, 2001). Therefore, we dissected bent cotyledon embryos and germinated them in auxin containing-medium to give rise to embryogenic clusters according to Mordhorst *et al.* (1998). No difference was seen in induction rate of embryogenic clusters compared to that of the wild-type. *Ep3-1* clusters were subcultured and remained embryogenic (data not shown), indicating that the absence of AtEP3 alone is not sufficient to impair somatic embryo development. We conclude that neither zygotic nor somatic embryos are impaired in their development in the absence of AtEP3 protein.

We have previously noted that the *AtEP3* promoter was also active during germination in the inner layers of the seed coat or in the endosperm at the location where the radicle protrudes (Passarinho *et al.*, 2001). When investigating seed germination in *ep3-1* we found an additional defect. It appeared that when put to germinate shortly after harvest, *ep3-1* seeds are non-dormant and can germinate without stratification (Table 2).

Line	Germination [a]	Total [b]	% germination [c]
ep3-1	81	111	73
Col-0	1	174	0.5

Table 2. Analysis of seed germination in the *ep3*-1 mutant.
One week-old or younger seeds were germinated on wet filter paper and counted after 5 days. [a] number of seeds germinated after 5 days. [b] total number of seeds put to germinate. [c] number of seeds germinated ÷ total number of seeds x 100

Next we investigated root and root hair development in young seedlings. Root elongation was faster in *ep3-1* seedlings than in the wild-type (Figure 5).

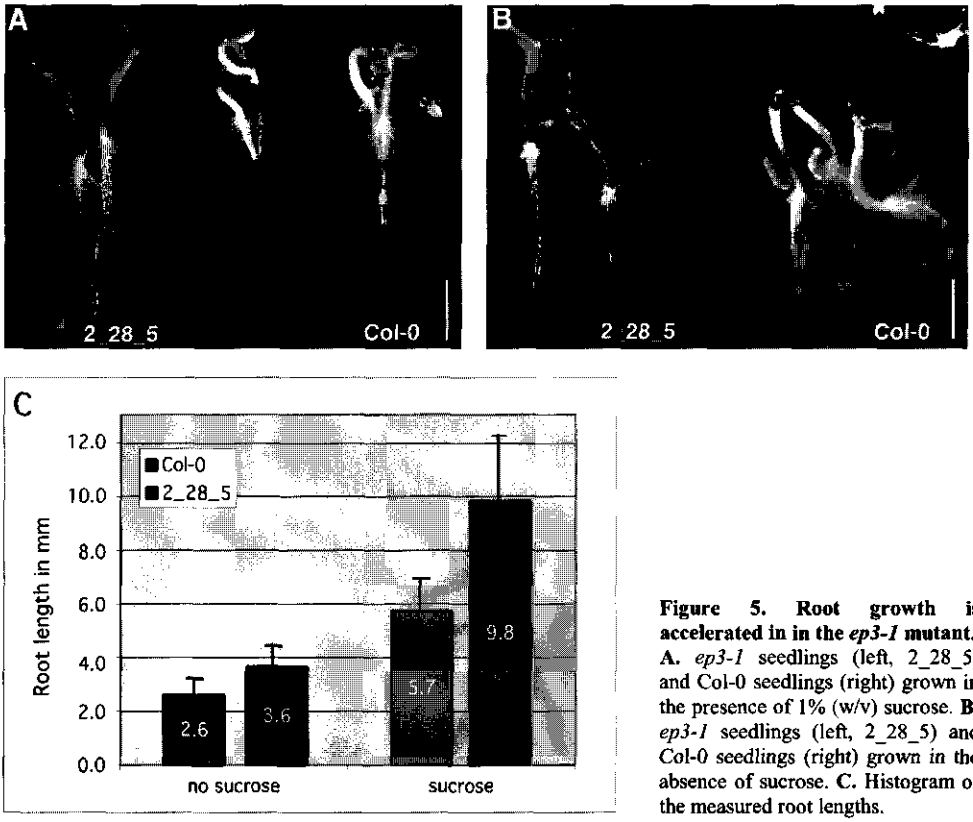

Figure 5. Root growth is accelerated in in the *ep3-1* mutant. **A.** *ep3-1* seedlings (left, 2_28_5) and Col-0 seedlings (right) grown in the presence of 1% (w/v) sucrose. **B.** *ep3-1* seedlings (left, 2_28_5) and Col-0 seedlings (right) grown in the absence of sucrose. **C.** Histogram of the measured root lengths.

When looking at 3 day-old seedlings germinated on regular germination medium (i.e. with 1% (w/v) sucrose) root length was almost double that of the wild-type (Figure 5A and C). Interestingly, in the absence of sucrose this difference was considerably reduced (Figure 5B

and C). The role of sucrose also appeared important in root hair development. In the presence of sucrose we observed that root hairs were formed closer to the root tip than in the wild-type, a difference that was noted as a decrease in the length of the elongation zone (Figure 6A-D). We also observed that root hairs were longer in *ep3-1* than in the wild-type (Figure 6A and B). However, in the absence of sucrose these differences were hardly visible (Figure 6E and F).

No anomaly was found in leaf morphology or in any of the aerial parts of the plant, although AtEP3 expression was also found in stipules and hydathodes (Passarinho *et al.*, 2001).

Discussion

To our knowledge, this work is the first example of a functional study based on a true chitinase mutant that we have named *ep3-1*. We have demonstrated that in homozygous plants of this line no *AtEP3* mRNA is transcribed as a result of a *dSpm* insertion in the second exon of the gene. Under normal growth conditions, the absence of AtEP3 chitinase mRNA did not affect embryo development nor did it result in major alterations in plant morphology. The subtle phenotypes we observed are in part in agreement with the expression pattern of the AtEP3 gene (Passarinho *et al.*, 2001).

First, inactivation of the *AtEP3* locus caused a clear reduction of pollen germination in vitro. Yet, this did not result in any fertility problems in planta. Such a defect may be difficult to observe in vivo since it would probably be complemented by stigma exudates, as we have seen in vitro. Together with sufficient remaining germinating pollen, this is probably enough for fertilization to occur normally. This defect in pollen germination correlates well with the observed expression of the *AtEP3* gene in maturing pollen grains and growing pollen tubes in planta (Passarinho *et al.*, 2001). The second phenotype we observed was an almost complete absence of seed dormancy. This is also consistent with the expression of the *AtEP3* gene in the endosperm cap or inner layers of the seed coat prior to radicle protrusion (Passarinho *et al.*, 2001). A third effect was observed during root hair development, where absence of *AtEP3* mRNA was reflected by an increase in root hair length and a decrease of the elongation zone, resulting in more "hairy" roots. Again this is in agreement with the expression of AtEP3 in growing root hairs (Passarinho *et al.*, 2001).

Although the only role of the EP3 chitinase known so far is during somatic embryogenesis (de Jong *et al.*, 1992) and in spite of the expression of AtEP3 in embryogenic cultures and not in

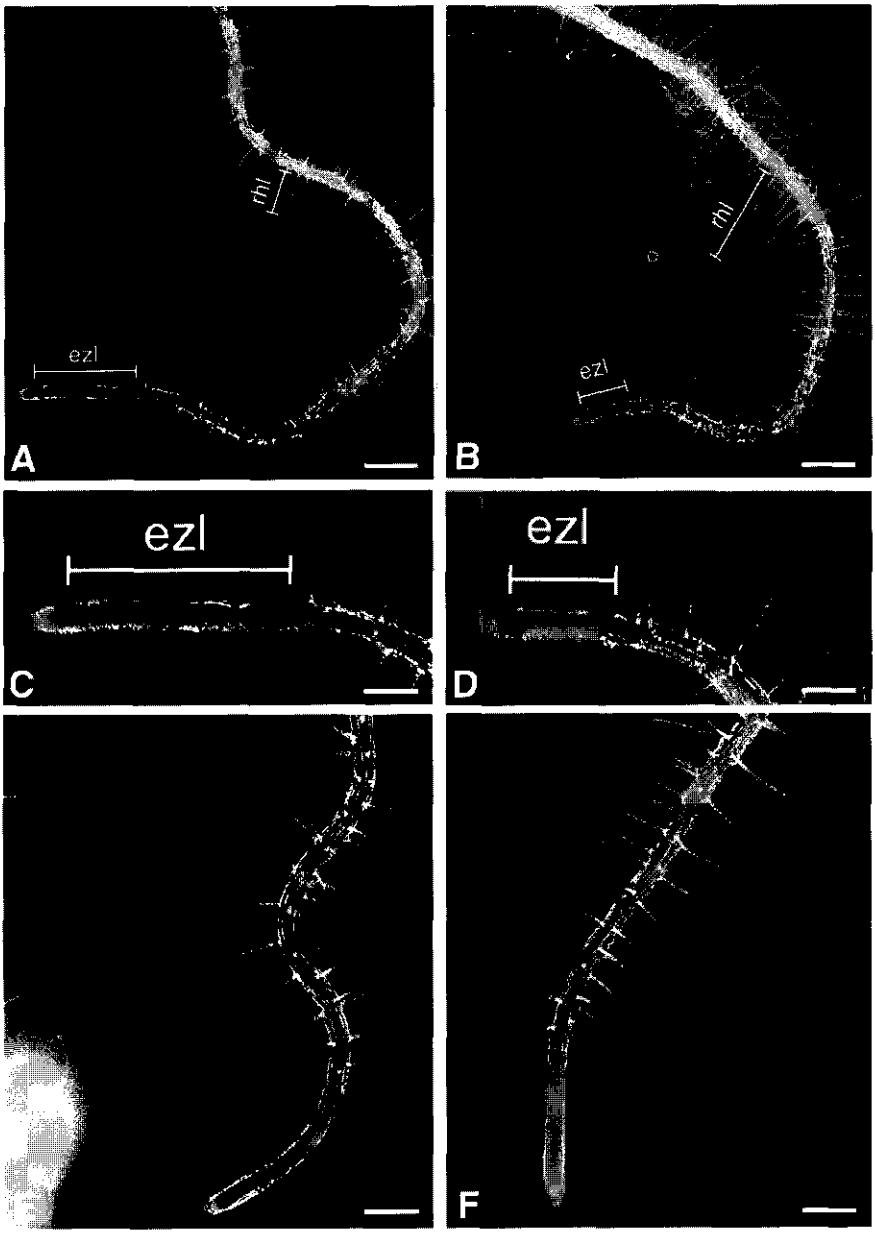

Figure 6. Root hair growth is disturbed in the *ep3-1* mutant.
A. Root of a Col-0 seedling grown in the presence of 1% (w/v) sucrose. **B.** Root of an *ep3-1* seedling grown in the presence of 1% (w/v) sucrose. **C.** Enlargement of the elongation zone from the root seen in A. **D.** Enlargement of the elongation zone from the root seen in B. **E.** Root tip and root hairs of a Col-0 seedling grown in the absence of sucrose. **F.** Root tip and root hairs of an *ep3-1* seedling grown in the absence of sucrose. *ezl* stands for elongation zone length, and rhl stands for root hair length. Bar = 1.25 mm in A and B, 0.5 mm in C and D and 0.75 mm in E and F.

non-embryogenic ones (Passarinho *et al.*, 2001), we did not observe any noticeable difference with the wild-type at this level. This suggests that the absence of AtEP3 chitinase alone is not sufficient to impair somatic embryo development, which also supports the hypothesis of Kragh *et al.* (1996) who proposed that the *ts11* mutant phenotype was caused by the absence of several chitinases. Chitinases other than EP3 could also rescue *ts11* embryos, which probably suffer a more general secretion defect (Baldan *et al.*, 1997; de Jong *et al.*, 1995). In *Arabidopsis* cultures that have no secretion defect, the effect of the absence of a single chitinase is most likely obscured by the presence of other chitinases. Apparently, this does not happen during pollen germination in vitro, which may be due to the absence of other chitinases. This could also explain the complementation of the pollen germination phenotype by stigma exudates that might contain other chitinases able to replace AtEP3. As a matter of fact, several chitinases were described to be present in the style of a number of plant species (Leung, 1992; Takakura *et al.*, 2000). An important question is what the biological function of chitinases in pollen development, seed dormancy and root hair formation is. While no proof is currently available, there are several interesting observations that suggest that chitinases may act through a class of cell wall proteoglycans designated as arabinogalactan proteins (AGPs). Van Hengel *et al.* (2001) have recently demonstrated that certain AGPs can be cleaved by endochitinases, suggesting that AGPs are a natural substrate for these enzymes. Furthermore, other studies have shown that certain AGPs were able to stimulate and guide pollen tube growth in vitro, suggesting an active role in pollen germination pollen tube growth (Cheung *et al.*, 1995; Wu *et al.*, 1995; Wu *et al.*, 2000). It is not known whether chitinases other than EP3 are able to cleave AGPs and modulate their action. However, during pollen germination and pollen tube growth, both chitinases and AGPs indeed co-localize. Interestingly, Lu *et al.* (2001) have shown that AGP expression is both qualitatively and quantitatively regulated during tomato seed germination. By blocking the activity of AGPs, using β-glucosyl Yariv, they did not observe any germination defect. However seed dormancy was not addressed in their study. In addition, in carrot and in *Arabidopsis*, AGPs and EP3 chitinase clearly co-localize at seed maturity (Passarinho *et al.*, 2001; van Hengel *et al.*, 1998a; van Hengel *et al.*, 1998b). However it is not clear how chitinases and AGPs may be involved in the maintenance of seed dormancy.

Seed dormancy is induced by the plant hormone abscisic acid (ABA) and most mutants that show reduced dormancy are impaired in ABA biosynthesis or sensitivity (Koornneef and Karssen, 1994; Leon-Kloosterziel *et al.*, 1996). Therefore, it will be interesting to see whether dormancy is reestablished in *ep3-1* seeds when germinated in the presence of ABA and see if

there is any link with ABA signaling.

A possible link between endochitinases and AGPs can also be made in the case of root and root hair development. AGPs are indeed localized on the root epidermal surface (Samaj *et al.*, 1999) and it was shown that blocking their action inhibits overall root length, epidermal root cell elongation, root cell numbers and root hair formation (Lu *et al.*, 2001; Willats and Knox, 1996). The nature of the defect observed in *ep3-1* plants together with these observations suggest that a mechanism in which AGPs and AtEP3 are involved is probably affected here as well, although we observe a promoting effect rather than the inhibitory ones observed by general interference with AGPs, using β-glucosyl Yariv. Therefore, we can only speculate that this reflects highly specific interactions between AGPs and the AtEP3 chitinase.

Another interesting observation was that the root and root hair phenotypes were only visible in the presence of sucrose, implying a different sensitivity to sucrose in *ep3-1* plants that results in promoting root and root hair growth. We did not analyze if there was any dosage effect or the influence of other sugar sources. However, this is clearly a next step for this work, especially in the light of a possible link between sucrose and ABA signaling (Laby *et al.*, 2000).

In conclusion, our data suggest that the AtEP3 chitinase is involved in a largely unknown signaling pathway, perhaps employing AGPs or AGP-derived GlcNAc-containing signal molecules and maybe coupled to sugar signaling. And although it still needs further confirmation, the nature of the phenotypes we observed in *ep3-1* plants is in line with a role in intercellular communication essential for processes such as somatic embryo development (Mordhorst *et al.*, 1997), pollen-stigma interactions (Pruitt, 1999), seed germination (Koornneef and Karssen, 1994) and root development (Scheres, 1997).

Materials and methods

Plant material

Arabidopsis thaliana (L.) Heynh wild-type and transgenic plants were grown under long day light conditions after germination on wet filter paper or on 0.5x MS salts (Murashige and Skoog, 1962), 1% (w/v) sucrose, 0.8% (w/v) agar. Seeds of the Sainsbury Laboratory Arabidopsis Transposants (SLAT lines; generated at the laboratory of Jonathan Jones, Sainsbury Laboratory, Norwich, UK) were obtained from The Nottingham Arabidopsis Stock Centre (Nottingham, UK). The wild-type ecotype *Columbia* (Col-0) was used as control.

DNA analysis

Genomic DNA was isolated from individual transgenic plants as well as from wild-type, according to Kozik *et al.* (1996). The *dSpm* insertion into the *AtEP3* gene was confirmed by PCR using *AtEP3* gene-specific primers (PP1, 5'-TTCGTCAGAGCTATGTTGTAGT CAGTTTGG-3' or PP6, 5'-CCACAAGGCGGTTTTGAAGGATATGACTGG-3') in combination with *dSpm*-specific primers (dSpm1, 5'-CTTATTTCAGTAAGAGT GTGGGGTTTTGG-3' or dSpm11, 5'-GGTGCAGCAAAACCCACACTTTTATTC-3' and the nested primer dSpm5, 5'-CGGGATCCGACACTCTTTAATTAACTGACACTC-3'). PCR products were directly sequenced, after purification using the High Pure PCR Product Purification kit (Roche Molecular Biochemicals, Mannheim, Germany).

RT-PCR analysis

Total RNA was isolated from flowers using TRIZOL® reagent (Invitrogen Life Technologies, Breda, The Netherlands) according to manufacturer's recommendations. RNA was DNAse-treated for 30 min at 37°C with 3 units RQ1 DNAse (Invitrogen Life Technologies) in 40 mM Tris-HCl pH 8.0, 10 mM NaCl, 6 mM $MgCl_2$ and 10 mM $CaCl_2$. After phenol/chloroform extraction and ethanol precipitation, the RNA was resuspended to a concentration of 500 ng μl^{-1}. Two µg DNAse-treated RNA were reverse-transcribed for 1h at 37°C, using 1 µg oligodT_{12}, 1mM dNTPs, 40 units RNAse out (Invitrogen Life Technologies) and 200 units M-MLV reverse-transcriptase (Invitrogen Life Technologies) in 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$ and 5 mM DTT. Presence of remaining genomic DNA was verified by a control reaction performed for each sample without reverse-transcriptase. After enzyme denaturation for 5 min at 95°C and a 20-fold dilution, 2 µl reverse-transcribed RNA were analyzed by PCR, using *AtEP3* gene-specific primers (PP1 and PP6, see above) and, as an internal standard for quantification, primers specific for the constitutive cyclophilin gene *ROC5* (Chou and Gasser, 1997; ROC5-5', 5'-TCTCTCTTCCAAATCTCC-3', and ROC5-3', 5'-AAGTCTCTCACTTTCTCACT-3'). PCR products were analyzed by agarose gel electrophoresis, Southern blotting and hybridization to radiolabeled gene-specific probes.

Phenotypic analysis

Seed set was analyzed by collecting about 10 siliques per plant starting from the fourth silique below the terminal inflorescence. Siliques were opened under a Nikon SMZ-2T binocular microscope and seeds were counted and classified according to their stage of

development: early aborted (white and tiny), late aborted (brown and shrunken) and fully developed.

Embryogenic capacity was analyzed in vitro by establishing embryogenic cultures using dissected immature zygotic embryos, as described by Mordhorst *et al.* (1998). The cultures were scored by visual observation starting from the third week after initiation. The formation of smooth green embryogenic clusters was noted weekly and compared with the wild-type.

Seed germination tests were performed on fresh seeds (maximum 1 week old) that were germinated on wet filter paper without stratification. The number of germinated seeds was counted 5 days later.

Root and root hair observations were carried out on young seedlings (up to 7 days old) grown vertically on 0.5x MS salts (Murashige and Skoog, 1962), 0.8% (w/v) agar with or without 1% (w/v) sucrose. Root length measurements were done directly on the seedlings, whereas elongation zone and root hair length were determined by computer on digital images of the roots.

Pollen in vitro germination tests were done essentially as described by Krishnakumar and Oppenheimer (1999).

Acknowledgements

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Chapter 7

Summarizing discussion

The work presented in this thesis deals with the role plant chitinases may have in development. Plant chitinases represent a large family of proteins of which the biological functions remain poorly understood. They are glycosyl hydrolases that catalyze the hydrolysis of chitin, a polymer of N-acetylglucosamine (GlcNAc). Since chitin is not found in plants but in the cell walls of most fungi, chitinases are thought to be involved in plant defense against fungal pathogens. A role in plant development was also suggested by several studies. We present here the first genetic evidence that a plant chitinase, the *Arabidopsis* AtEP3 class IV endochitinase, is indeed involved in development. While the biochemical details of the action of this chitinase are not known, they may involve cell to cell signaling.

The study of the *Arabidopsis* AtEP3 chitinase is based on previous work carried out in our laboratory that had shown the important role of the EP3 carrot chitinase during somatic embryo development (de Jong *et al.*, 1992). Unfortunately studying the function the EP3 chitinase in carrot was hindered by the fact that it belongs to a family of at least five members that are so closely related in sequence that they are not serologically distinguishable (de Jong *et al.*, 1995; Kragh *et al.*, 1996). It was therefore decided to investigate the role the EP3 chitinase could play in development in another plant species. The model plant *Arabidopsis thaliana* was the obvious choice based on the small size of its genome and the growing number of genetic tools available. The identification and the study of the *Arabidopsis* ortholog of the carrot EP3 chitinase gene are the focus of this thesis. The main goal was to identify an *Arabidopsis* knockout mutant for the *AtEP3* gene and perform a functional analysis in order to unravel its role during embryogenesis.

Reverse Genetics.

We have succeeded to identify a knockout mutant for the AtEP3 chitinase and to initiate the analysis of its function. In the course of this analysis we came across several difficulties inherent to reverse genetic approaches.

The first difficulty resides in the identification of a phenotype. Many gene knockouts do not generate visible phenotypes under normal growth conditions (Bouche and Bouchez, 2001). It is only based on the detailed knowledge of a gene expression pattern that one can direct the search for a phenotype. The subtle phenotypes we identified in *ep3-1* plants would have been easily missed on a large-scale analysis. This was probably the case in Chapter 4, where we conducted a phenotypic analysis on a large plant population that was not genetically defined. Our attention was mainly focused on embryo development and fertility, but as we saw in *ep3-1* plants for which we knew that the *AtEP3* mRNA was not produced, there is nothing visible at the plant level. We did not perform pollen in vitro germination tests on the whole plant population, neither did we analyze seed dormancy which needs to be specifically investigated. The difficulty in detecting such phenotypes also comes from the problem of gene redundancy. The absence of a given protein can easily be masked by other proteins of the same family. Therefore it is only when placed in conditions where these proteins would not be present that we can possibly identify a phenotype due to the absence of a single member of a family, such as in the pollen in vitro germination test. This is not always possible and it may mean that one has to make multiple knockouts within a family to be able to visualize a phenotype. Now that the sequencing of the *Arabidopsis* genome has reached completion standardized methods are arising to be able to perform detailed phenotypic analyses on a large-scale. This implies the definition of very specific stages of development together with specific methods of observations with defined criteria for different processes (Boyes *et al.*, 2001).

The second difficulty encountered in reverse genetic approaches is directly linked to the nature of the plant collection used for the screening (Chapter 4) and reflects the mistakes of an emerging technology. A large number of mutagenized populations are available and it is possible to screen most of them by high throughput PCR methods. However, not all of them are suitable for reverse genetic approaches. The design of such a collection is indeed of great influence on the subsequent analysis of a knockout line. The number of inserted elements should be very small, ideally limited to one. These elements should be stable and possess a readily usable selection marker allowing a fast an easy selection at the plant level. This also means that very large plant populations are required and implies the setup of large facilities

such as the *Arabidopsis* Knockout Facility at the University of Wisconsin-Madison (Sussman *et al.*, 2000). Therefore new methodologies are needed and are arising in order to provide powerful tools to the research community that will allow to simultaneously study the function of complete gene families, such as chitinases.

Is AtEP3 the *Arabidopsis* ortholog of the carrot EP3 chitinase?

We have identified an *Arabidopsis* gene that based on sequence similarity is the ortholog of the carrot EP3 endochitinase (Chapter 2). The *AtEP3* gene is a single copy gene that is most similar to the carrot *EP3-3* gene, that encodes the isoform that is the most active in the rescue of *ts11* mutant embryos (Kragh *et al.*, 1996). Both chitinases are serologically related and have very similar biochemical properties (specific activity and pH optimum). In addition, the native encoded AtEP3 chitinase was able to rescue the carrot mutant cell line *ts11*. The knockout of the *Arabidopsis AtEP3* gene did not result in a defect in embryo development as we had expected based on the work previously done in carrot (de Jong *et al.*, 1992). Instead, we found subtle phenotypes during pollen development, seed germination and root hair development. Another explanation for the fact that we do not find a phenotype during *Arabidopsis* zygotic and somatic embryogenesis could be that *ts11* is not a chitinase mutant but has a more general secretion defect affecting other chitinases as well (Baldan *et al.*, 1997; de Jong *et al.*, 1995). This was confirmed by the fact that other chitinases than EP3 can rescue the *ts11* mutant (Kragh *et al.*, 1996). It is therefore very likely that, as in the embryo mutant *ts11*, we can only have a visible effect on somatic embryogenesis if more than one chitinase is absent from the culture medium. Therefore it is possible that the true functional ortholog is encoded by one of the *Arabidopsis* sequences with less sequence homology to the carrot genes. At the time we performed our database searches the sequencing of the *Arabidopsis* genome was still far from completion. We later realized that there are more related chitinase genes than we found initially (Chapter 1).

All three phenotypes found are in agreement with the expression pattern of the gene that was analyzed in detail by RT-PCR and by means of promoter::GUS fusions. It appeared to be rather similar to the expression found in carrot during somatic embryogenesis (van Hengel *et al.*, 1998a) but there were some discrepancies as well. However, we should keep in mind that in carrot, the EP3 family members are serologically indistinguishable and the probes used for RNA in situ hybridization did not allow to distinguish between the different *EP3* genes (van Hengel *et al.*, 1998a). All isoforms might therefore have been looked at simultaneously.

Furthermore, in *Arabidopsis* we could perform a more detailed expression analysis at the tissue level that may extend the results found in carrot, especially during vegetative development. This may also be true for the phenotypes we found in *Arabidopsis*. In carrot, it was not possible to conduct a similar mutant analysis. It is therefore unknown whether the carrot EP3 chitinase is also involved in processes other than embryogenesis. The results of the RT-PCR analysis carried out in carrot tend to indicate that this may be the case since the carrot EP3 mRNA was also found in storage roots, flowers and in imbibed mature seeds (van Hengel *et al.*, 1998a).

On the occurrence of gametophytic gene expression programs during somatic embryogenesis.

Based on the specific expression pattern of the *AtEP3* gene in embryogenic suspension cultures and its expression during pollen development, we have investigated the occurrence of gametophytic gene expression programs during somatic embryogenesis. Highly specific GUS markers for the male and the female gametophytes were included in this study and this revealed that all gametophytic markers tested were expressed during somatic embryogenesis in a temporally and spatially regulated manner. Such a specific regulation confirmed previous observations that were made in carrot embryogenic suspension cultures, where it was also shown that various cell types co-exist and have different fates and functions (McCabe *et al.*, 1997; Toonen *et al.*, 1994). For instance, the immunolocalization of the specific arabinogalactan (AGP) epitope JIM8 (Pennell *et al.*, 1991) in carrot embryogenic cultures revealed that a certain type of cells first possesses and then looses the JIM8 epitope. Further work suggested that it was the JIM8-negative cells that develop from these JIM8-positive cells that are competent to form embryos in culture (McCabe *et al.*, 1997). The JIM8-positive cells present in these cultures would then mimic a postulated nursing role of the seed endosperm allowing the formation and the development of the embryo. The localization of the JIM8 epitope in the cell wall of gametophytic cells in planta - e.g. *Brassica* sperm and *Lilium* sperm and generative cells (Southworth and Kwiatkowski, 1996); micropyle of *Amaranthus hypochondriacus* ovules (Coimbra and Salema, 1997) – is also well established following earlier observations of its occurrence in *Brassica* in the nucellar epidermis, synergid cells, the egg cell and young embryos (Pennell *et al.*, 1991). These observations suggest that at least a number of pathways are shared between gametogenesis and embryogenic cell formation in

vitro. Whether the gene products have the same role in both processes remains to be elucidated, as well as its significance.

AtEP3 is involved in seed dormancy, pollen and root hair development; is there a common mechanism?

In Chapter 5 and 6 we have shown that the AtEP3 chitinase is involved in pollen and root hair development as well as in seed dormancy. These observations were in general agreement with the expression pattern of the *AtEP3* gene. One can wonder about the common denominator in the mechanism(s) by which the AtEP3 chitinase is involved in these three developmental processes without obvious correlation.

The expression of the *AtEP3* gene in mature pollen and growing pollen tubes and the pollen in vitro germination defect we observed imply the presence of a plant substrate in the direct environment of the mature pollen grain. AtEP3 being a secreted chitinase, such a substrate might be found in the locule, the stigma or in the transmitting tract of the style during pollen tube elongation. We have seen that the reduced pollen germination was only visible in vitro, without the influence of surrounding tissue. We have also observed that stigma exudates were able to mask the absence of AtEP3 chitinase in vitro. This also explains the absence of a phenotype in planta. As in embryogenic cultures, other chitinases produced by the stigma are most likely compensating the absence of AtEP3. This suggests that AtEP3 and probably other chitinases are active in the pollen grain environment. They most likely participate to pollen germination and the subsequent elongation of the pollen tubes through the processing of a substrate present in the transmitting tract. So far, the nature of this substrate is unknown. Interestingly, arabinogalactan proteins (AGPs) have been identified in pollen and transmitting tract of several plant species (e.g. Cheung *et al.*, 1995; Du *et al.*, 1996; Gerster *et al.*, 1996; Lind *et al.*, 1994). It was proposed that they could promote pollen germination, pollen tube growth and serve as chemoattractants for their guidance (Wu *et al.*, 1995). From the work of van Hengel *et al.* (2001) we know that the carrot EP3 chitinase is able to cleave specific AGPs in vitro. AGPs co-localize as well with the presence of the enzyme in carrot, and after incubation with the EP3 chitinase their promoting effect on somatic embryogenesis (Kreuger and van Holst, 1993) is enhanced. These results strongly suggest that GlcNAc-containing AGPs could be a substrate for the EP3 chitinase. However, we do not know whether the AGPs involved in pollen tube growth in tobacco (Cheung *et al.*, 1995) contain GlcNAc residues and therefore whether they could be a substrate for chitinases.

We also do not know whether *Arabidopsis* stylar AGPs can be processed by chitinases. Therefore we can only speculate on a possible link between the AtEP3 chitinase and AGPs of the transmitting tissue that could be at the heart of signaling events eventually leading to the growth of a pollen tube.

The analysis of the expression pattern of the AtEP3 gene also revealed that after the pollen tube enters the receptive synergid its content is released into the latter along with the two sperm cells and even reaches the central cell, as illustrated by the observed GUS stainings (Chapter 2). This suggests that the pollen tube does not only supply the two sperm cells to the female gametophyte but carries a number of other factors that could favor the fertilization event itself and/or the very first steps of embryo development. The AtEP3 chitinase could be one of these factors, providing there is a substrate such as GlcNAc-containing AGPs that is present in the embryo sac or that is carried along with the chitinase from the pollen tube. The studies of Coimbra and Salema (1997) and Pennell *et al.* (1991) indicate that AGPs are present in the female gametophyte prior to fertilization. It is unknown whether these specific AGPs contain cleavage sites for the EP3 chitinase.

The second defect we observed in the *ep3-1* mutant was also very subtle and was only identified based on the expression of the *AtEP3* gene upon germination in the endosperm cap or the inner layers of the seed coat. We did not observe any reduction in seed germination but instead reduced seed dormancy was observed. This suggests a role of the AtEP3 chitinase in maintaining the dormancy state in wild-type seeds. The mechanism by which a chitinase could be involved here is totally obscure. The plant hormone abscisic acid (ABA) is known for inducing seed dormancy and most mutants that show reduced dormancy are impaired in ABA biosynthesis or sensitivity (Koornneef and Karssen, 1994; Leon-Kloosterziel *et al.*, 1996). This may point to a role of the AtEP3 chitinase in a pathway correlated to ABA or in some unknown pathway that also controls seed dormancy. Therefore, it would be interesting to see whether *ep3-1* seeds remain non-dormant in the presence of ABA. A possible mechanism could also involve AGPs, as proposed for pollen development. Lu *et al.* (2001) have indeed shown that AGP expression is both qualitatively and quantitatively regulated during seed germination. By blocking the activity of AGPs they did not observe any germination defect. Seed dormancy was not addressed in their study. AGPs are also present in *Arabidopsis* seeds (van Hengel *et al.*, 1998b), but as for other plant species it is unknown whether, as in carrot, these AGPs contain cleavage sites for the EP3 chitinase. How the processing of a hypothetical GlcNAc-containing AGP would influence seed dormancy is not known.

Finally, we observed a defect in root hair development that could be in line with the pollen tube growth observations, since root hair and pollen tube elongation both occur through tip growth. This is clearly illustrated in the *Arabidopsis tip1* mutant that is impaired both in pollen tube growth and root hair elongation (Schiefelbein *et al.*, 1993). In *ep3-1* plants, we see a root hair phenotype opposite to the one of the *tip1* mutant. Root hairs are longer than in the wild-type, and in C4-5 plants where the *AtEP3* mRNA level is 10% that of the wild-type root hairs are swollen. The significance of this difference in phenotype is unknown, but in the C4-5 line other chitinase genes may have been affected as well as the *AtEP3* gene. The precise combination of chitinases remaining expressed may have resulted in such a difference. The nature of the root hair phenotype suggests that the AtEP3 chitinase may act through a different mechanism in root hair than in pollen development. If the AtEP3 chitinase is involved in tip growth it may have both stimulating effects as seen in pollen tube elongation and inhibitor effects as seen in root hair growth. AGPs are present on the root surface (Samaj *et al.*, 1999) and it was shown that blocking the action of AGPs inhibits overall root length, epidermal root cell elongation, root cell numbers and root hair formation (Lu *et al.*, 2001; Willats and Knox, 1996). The nature of the defect observed in *ep3-1* plants together with these observations is not in contradiction with a mechanism involving specific combinations of AGPs and chitinases. We will need to look specifically at the roots of *ep3-1* plants to describe the root hair phenotype in more detail. Other indications for a function in root hair development arise from the observations made in the *Rhizobium*-legume symbiosis, in which lipochitinoligosaccharides (LCOs) produced by the bacterium are responsible for morphological changes occurring in the root hair among which elongation (Dazzo *et al.*, 1996). We also know from previous work of de Jong *et al.* (1993) that LCOs are able to rescue *ts11* mutant embryos. This suggests that similar signals are involved in both morphological processes of root hair deformation prior to forming a root nodule and the formation of an embryo. In addition, plant chitinases are able to process LCOs (Schultze *et al.*, 1998) that as the AGPs described in van Hengel *et al.* (2001) contain GlcNAc residues. Taken together our observations support the hypothesis that GlcNAc-containing molecules such as AGPs that are processed by the EP3 chitinases are involved in a developmental process such as tip growth.

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Summary

Chitinases are enzymes that are capable of catalyzing the hydrolysis of chitin, a homopolymer of N-acetylglucosamine. Chitin is the main constituent of the exoskeleton of insects, of crustacean shells and of the cell wall of many fungi but is absent in plants. This led to the commonly accepted hypothesis that plant chitinases are involved in defense against pathogens with chitin in their cell wall such as certain classes of fungi. Yet their role is not restricted to responding to pathogen attacks since plant chitinases are also induced by various types of stress, for instance after treatment with heavy metals or after UV irradiation. Chitinases can also be induced by plant hormones and they have been associated with a number of developmental processes, most notably in embryogenesis and during pollination. In addition, some chitinases may play a role in defense as well as in development, depending on their expression at particular stages in the plant life cycle. Plant chitinases belong to a relatively large protein family, which has hampered attempts to gain a better understanding of their role. A detailed study of individual chitinases is a prerequisite to unravel their precise role as well as to determine the function the different members of the five classes in which plant chitinases are subdivided.

In this thesis, we have addressed the role of one particular chitinase, AtEP3, in the model plant *Arabidopsis thaliana*. The work presented illustrates some of the difficulties inherent to the study of individual genes that belong to fairly large gene families. Chapter 1 gives a detailed overview of all chitinase genes present in the *Arabidopsis* genome. The genomic distribution and the sequences of these genes revealed interesting evolutionary relationships between the different classes. We discuss the possible significance of some of their sequence characteristics in light of their predicted role and propose a number of functions based on chitinases studied in other plants.

In Chapter 2, we present an elaborate analysis of the expression pattern of the *AtEP3* gene. The expression pattern of the *AtEP3* chitinase gene suggested possible functions in somatic embryo development, pollen maturation and/or germination, pollen tube growth, seed germination and root hair growth. All of these aspects have been looked

at when searching for morphological aberrations (Chapters 5 and 6). The analogy between the expression of the gene found in pollen and in embryogenic cultures suggested there may be a correlation between gametogenesis and embryogenic cell formation. This notion was further taken into account by a study of GUS markers for specific cells of the female gametophyte and for the male gametophyte (Chapter 3). This work clearly indicated that a number of genetic programs specific for both gametophytes *in planta* are reproduced in tissue culture and that they are regulated in a spatially and temporally manner.

In Chapter 4, we were confronted with some of the pitfalls of reverse genetics. We performed a molecular and phenotypic analysis of several mutant plants in which the AtEP3 gene had been disrupted. However, we did not succeed in identifying any phenotype that could be directly linked to the absence of the AtEP3 chitinase. This was mainly due to the genetic instability of the material we studied, combined with the growth conditions in which we performed our analysis. It became clear from this work that small errors introduced while generating the available mutant plant collections can prevent the recovery of the desired individual mutant plants. In addition, the number of elements inserted for mutagenesis greatly influences the ease by which the phenotypic analysis of the mutant plants can be performed since multiple insertions can lead to several unlinked phenotypes.

In Chapter 5, we describe several transgenic lines in which the expression levels of the *AtEP3* gene had been manipulated. An increase in AtEP3 expression did not result in any visible change in plant morphology, nor in embryogenic potential *in vitro*. However, a reduction of AtEP3 expression to 10% the level of the wild-type resulted in a defect in root hair morphology. Similarly, complete knockout of the gene produced a root hair phenotype in a mutant plant now renamed *ep3-1* (Chapter 6). Both phenotypes suggest a role for AtEP3 in root hair formation. Complete absence of *AtEP3* mRNA also gave rise to the direct germination of fresh seeds without prior stratification, indicating that the chitinase could also be involved in the maintenance of seed dormancy. An additional defect was a strong reduction of pollen development *in vitro*. Surprisingly, the reduced pollen germination phenotype in the *ep3-1* mutant could be compensated by stigma exudates *in vitro*. This makes it very unlikely that such a phenotype could ever be observed *in planta*. The absence of AtEP3 in pollen is most likely compensated by the presence of other chitinases in the stigma. This is probably the case in embryogenic cultures as well, where the absence of a single

chitinase might not be sufficient to hinder embryo development. Previous work done in carrot is in line with these observations.

We have previously proposed the involvement of arabinogalactan proteins (AGPs) as a possible substrate for the AtEP3 chitinase. We base this hypothesis on the findings that carrot EP3 chitinases can cleave specific AGPs and that as a result the promotive effect of these now "cleaved" AGPs on somatic embryo development is enhanced. Second, AGPs are often found at the same location as chitinases and finally AGPs have been shown by others to be involved in pollen and root development as well as in seed germination. Taken together these observations suggest a role for the AtEP3 chitinase in intercellular communication through N-acetylglucosamine-containing signal molecules. The work presented in this thesis provides the groundwork that is essential to address the role of plant chitinases by molecular, genetic and biochemical means.

Samenvatting

Chitinases zijn enzymen die de hydrolyse van chitine, een homopolymeer van N-acetylglucosamine, kunnen katalyseren. Chitine is het belangrijkste bestanddeel van het exoskelet van insecten, van schaaldieren en van de celwand van vele schimmels maar is afwezig in planten. Dit leidde tot de algemeen geaccepteerde hypothese dat plantchitinases betrokken zijn bij het bescherming van planten tegen pathogenen met chitine in hun celwand zoals bepaalde klassen van schimmels. Maar toch is hun rol niet beperkt tot het reageren tegen pathogenenaanvallen aangezien plantchitinases ook door diverse types stress kunnen worden geïnduceerd, bijvoorbeeld na behandeling met zware metalen of na UV-straling. Chitinases kunnen eveneens door planthormonen worden geïnduceerd en worden geassocieerd met een aantal ontwikkelingsprocessen, voornamelijk embryogenese en polinisatie. Bovendien, zouden sommige chitinases een rol kunnen spelen in bescherming en in ontwikkeling, afhankelijk van hun expressie in specifieke stadia van het plantlevenscyclus. Plantchitinases behoren tot een vrij grote eiwitfamilie en dit heeft het verkrijgen van een beter inzicht in hun rol belemmerd. Een gedetailleerde studie van individuele chitinases is vereist om hun nauwkeurige rol te ontrafelen evenals om de functie te bepalen van de verschillende leden van de vijf klassen waarin plantchitinases worden onderverdeeld.

In dit proefschrift hebben wij ons gericht op de rol van één specifiek chitinase, AtEP3, in de model plant *Arabidopsis thaliana*. Het hier beschreven werk illustreert enkele van de moeilijkheden die behoren bij de studie van individuele genen die onderdeel zijn van vrij grote genfamilies. Hoofdstuk 1 geeft een gedetailleerd overzicht van alle chitinasegenen aanwezig in het *Arabidopsis* genoom. De genomische verdeling en de sequenties van deze genen brachten interessante evolutieverbanden tussen de verschillende klassen aan het licht. De mogelijke betekenis van sommige van hun sequentiekenmerken in relatie tot hun voorspelde rol wordt besproken en een aantal functies die worden gebaseerd op bekende chitinases uit andere planten worden voorgesteld.

In Hoofdstuk 2, stellen wij een gedetailleerde analyse van het expressiepatroon van het gen *AtEP3* voor. Het expressie patroon van dit gen duidt op mogelijke functies in de somatische embryo-ontwikkeling, stuifmeelrijping en/of ontkieming, de groei van de stuifmeelbuis, zaad ontkieming en de groei van wortelharen. Elk van deze aspecten zijn bekeken tijdens het zoeken naar morfologische afwijkingen (Hoofdstukken 5 en 6). De analogie tussen de expressie van het gen in stuifmeel en in embryogene culturen impliceert een mogelijke correlatie tussen gametogenese en embryogene celvorming. Dit werd verder onderzocht met behulp van GUS markers voor specifieke cellen van de vrouwelijke gametophyte en voor de mannelijke gametophyte (Hoofdstuk 3). Dit werk wees duidelijk uit dat een aantal genetische programma's specifiek voor beide gametophytes *in planta* in de weefselkweek worden gereproduceerd. Deze programma's zijn ook in tijd en ruimte gereguleerd.

In Hoofdstuk 4, werden wij geconfronteerd met enkele valkuiten van 'reverse genetics'. Wij voerden een moleculaire en fenotypische analyse van verschillende mutantplanten uit waarbij het gen *AtEP3* werd uitgeschakeld. Een fenotype dat direct met de afwezigheid van de AtEP3 chitinase zou kunnen worden verbonden werd echter niet geïdentificeerd. Dit is hoofdzakelijk toe te schrijven aan de genetische instabiliteit van het bestudeerde materiaal, gecombineerd met de groeiomstandigeden waarin de analyse werden uitgevoerd. Uit dit werk werd duidelijk dat kleine fouten geïntroduceerd tijdens het produceren van de beschikbare collecties van mutantplanten het verkrijgen van de gewenste individuele mutantplanten kan verhinderen. Bovendien, beïnvloedt het aantal elementen die voor mutagenese worden ingebracht zeer het gemak waarmee de fenotypische analyse van de mutantplanten kan worden uitgevoerd, aangezien veelvoudige inserties tot verschillende onafhankelijke fenotypes kunnen leiden.

In Hoofdstuk 5, worden verschillende transgene lijnen beschreven waarin het expressieniveau van het gen *AtEP3* is gemanipuleerd. Een verhoging van AtEP3 expressie resulteerde niet in een zichtbare verandering in de morfologie van de planten, noch in embryogene vermogen *in vitro*. Een vermindering van AtEP3 expressie tot 10% van het niveau van het wild-type resulteerde daarentegen in een defect in de morfologie van wortelharen. Zo ook, veroorzaakte de volledige 'knock-out' van het gen een fenotype in wortelharen in de mutantplant nu *ep3-1* genoemd (Hoofdstuk 6). Beide fenotypes wijzen op een rol voor AtEP3 in de vorming van het wortelhaar. De volledige afwezigheid van *AtEP3* mRNA leidde ook tot de directe ontkieming van

verse zaden zonder voorafgaande stratificatie. Dit wijst erop dat het AtEP3 chitinase ook in het onderhoud van zaadkiemrust geïmpliceerd zou kunnen zijn. Een extra defect was een sterke vermindering van de stuifmeelontwikkeling *in vitro*. Verrassend genoeg kon het verminderde *in vitro* stuifmeelontkieming in de mutant *ep3-1* door stigma afscheidingen gecompenseerd worden. Dit maakt het zeer onwaarschijnlijk dat een dergelijk fenotype ooit *in planta* kan worden waargenomen. De afwezigheid van AtEP3 in stuifmeel wordt waarschijnlijk door de aanwezigheid van andere chitinases in het stigma gecompenseerd. Dit is vermoedelijk ook het geval in embryogene culturen, waar de afwezigheid van één enkele chitinase niet zou kunnen volstaan om de embryo-ontwikkeling te belemmeren. Voorgaand werk dat in de wortel werdt uitgevoerd stemt met deze observaties overeen.

Wij hebben eerder de betrokkenheid van arabinogalactan proteïnen (AGPs) als een mogelijk substraat voor het AtEP3 chitinase voorgesteld. Deze hypothese wordt gebaseerd op de bevindingen dat wortel EP3 chitinases specifieke AGPs kunnen splitsen en dat dientengevolge het stimulerende effect van de nu 'gesplitste' AGPs op somatisch embryo-ontwikkeling wordt verhoogd. Verder, worden AGPs vaak gevonden op de zelfde plaats als chitinases en het is door anderen aangetoond dat AGPs in stuifmeel en wortelontwikkeling evenals in zaadontkieming zijn geïmpliceerd. Tezamen genomen deze observaties duiden op een rol voor het AtEP3 chitinase in intercellulaire communicatie door N-acetylglucosamine-bevatende signaalmoleculen. Het werk dat in deze proefschrift wordt beschreven legt de basis die essentieel is om het onderzoek naar het de rol van plantchitinases door middel van moleculaire, genetische en biochemische middelen aan te pakken.

Résumé

Les chitinases sont des enzymes capables de catalyser l'hydrolyse de la chitine, un homopolymère de N-acetylglucosamine. La chitine est le constituent principal Du squelette externe des insectes, des crustacées et de la paroi cellulaire de nombreuses moisissures, mais elle est absente des plantes. Ceci a mené à l'hypothèse généralement acceptée que les chitinases de plantes sont impliquées dans la défense contre des agents pathogènes contenant de la chitine dans leur paroi cellulaire telles que certaines classes de moisissures. Pourtant leur rôle n'est pas limité à répondre aux attaques de pathogènes puisque les chitinases de plantes sont également induites par divers types de stress, comme par exemple après traitement par des métaux lourds ou après irradiation aux ultra-violets. Les chitinases peuvent également être induites par des hormones végétales et ont été associées à un certain nombre de processus du développement, plus particulièrement l'embryogenèse et la pollinisation. En outre, certaines chitinases peuvent jouer un rôle aussi bien dans la défense des plantes que dans leur développement en fonction des stades spécifiques auxquels elles s'expriment pendant le cycle de vie de la plante. Les chitinases de plantes appartiennent à une relativement grande famille de protéines, ce qui a entravé de nombreuses tentatives visant à acquérir une meilleure compréhension de leur rôle. Une étude détaillée de chaque chitinase est nécessaire pour déterminer leur rôle précis ainsi que la nature des relations entre les différents membres des cinq classes dans lesquelles les chitinases de plante sont subdivisées.

Dans cette thèse, nous avons adressé le rôle de la chitinase AtEP3 chez la plante modèle *Arabidopsis thaliana*. Le travail présenté illustre certaines des difficultés inhérentes à l'étude de gènes qui appartiennent à une grande famille. Le Chapitre 1 donne une vue d'ensemble de tous les gènes de chitinase présents dans le génome d'*Arabidopsis*. La distribution génomique et les séquences de ces gènes ont révélé des relations intéressantes en termes d'évolution entre les différentes classes de chitinases. Nous discutons la signification possible de certaines de leurs caractéristiques à la lumière de leur rôle prédit et proposons un certain nombre de fonctions basées sur des chitinases étudiées chez d'autres plantes.

Dans le Chapitre 2, nous présentons une analyse détaillée du profil d'expression du gène *AtEP3*. Les résultats de cette analyse ont suggéré des fonctions possibles dans le développement de l'embryon *in vitro*, la maturation et/ou la germination du pollen, la croissance des tubes polliniques, la germination de la graine et la croissance des poils racinaires. Tous ces aspects ont été abordés en recherchant des anomalies morphologiques (Chapitres 5 et 6). L'analogie entre l'expression du gène dans le pollen et dans les cultures embryogènes suggère une corrélation possible entre la gamétogenèse et la formation de cellules embryogènes *in vitro*. Cette notion a été prise en considération par une étude de marqueurs GUS pour des cellules spécifiques du gamétophyte femelle et pour le gamétophyte mâle (Chapitre 3). Ce travail a clairement indiqué que certains des programmes génétiques spécifiques pour les deux gamétophytes *in planta* sont reproduits en culture *in vitro*. Ces programmes sont également régulés spacialement et temporellement.

Dans le Chapitre 4, nous avons été confrontés avec certains des pièges de la génétique inverse. Nous avons exécuté une analyse moléculaire et phénotypique de plusieurs plantes mutantes pour lesquelles le gène *AtEP3* a été interrompu. Cependant, nous n'avons pas réussi à identifier un phénotype qui pourrait être directement lié à l'absence de chitinase AtEP3. Ceci est principalement dû à l'instabilité génétique du matériel que nous avons étudié, ainsi qu'aux conditions de croissance des plantes dans lesquelles nous avons exécuté notre analyse. Cette étude a clairement montré que d'infimes erreurs introduites au cours de la production des collections de mutants, peut empêcher la récupération de la plante mutante recherchée. En outre, le nombre de transgènes influence considérablement la facilité par laquelle l'analyse phénotypique des plantes mutantes peut être exécutée puisque des insertions multiples de transgènes peuvent mener à plusieurs phénotypes indépendants les uns des autres.

Dans le Chapitre 5, nous décrivons plusieurs lignées transgéniques dans lesquelles le niveau d'expression du gène *AtEP3* a été manipulé. Une augmentation de l'expression d'*AtEP3* n'a eu aucune conséquence visible sur la morphologie de la plante, ni sur le potentiel embryogène *in vitro*. Cependant, une réduction de l'expression d'*AtEP3* à 10% du niveau du type sauvage a résulté en un défaut dans la morphologie des poils racinaires. De même, le KO complet du gène a produit un phénotype au niveau des poils racinaires chez une plante mutante maintenant nommée *ep3-1* (Chapitre 6). Ces deux phénotypes suggèrent un rôle pour AtEP3 dans la formation des poils racinaires. L'absence complète d'ARNm d'*AtEP3* a également provoqué la germination directe de

graines fraîches sans vernalisation antérieure, indiquant que la chitinase pourrait également être impliqué dans le maintien de la dormance de la graine. Un défaut supplémentaire a été observé, correspondant à une forte réduction du développement du pollen *in vitro*. Etonnamment, le phénotype de réduction de germination du pollen *in vitro* dans le mutant *ep3-1* a pu être compensé par des exsudats de pistil. Ceci rend l'observation d'un tel phénotype *in planta* très peu probable. L'absence d'AtEP3 dans le pollen est très probablement compensée par la présence d'autres chitinases dans le pistil. C'est probablement le cas aussi dans les cultures embryogènes où l'absence d'une seule chitinase pourrait ne pas être suffisante pour gêner le développement de l'embryon. Des travaux effectués chez la carotte sont en conformité avec ces observations.

Nous avons précédemment proposé la participation de protéines contenant de l'arabinogalactane (AGPs) comme substrat possible pour la chitinase AtEP3. Nous basons cette hypothèse sur le fait que les chitinases EP3 de la carotte peuvent cliver des AGPs spécifiques et qu'en conséquence l'effet promoteur de ces AGPs, maintenant clivés, sur le développement de l'embryon somatique est accru. En second lieu, les AGPs sont souvent trouvés au même endroit que les chitinases et finalement il a été montré que les AGPs sont impliqués dans le développement du pollen et de la racine ainsi que dans la germination de la graine. Toutes ces observations suggèrent un rôle pour la chitinase AtEP3 dans la communication intercellulaire par l'intermédiaire de molécules signal contenant de l'N-acetylglucosamine. Le travail présenté dans cette thèse fournit la trame de fond essentielle pour étudier le rôle des chitinases de plantes par des moyens moléculaires, génétiques et biochimiques.

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Paul

Curriculum Vitae

Paul Alexandre Passarinho was born on 20 April 1970 in Condrieu, France. In 1989, he obtained his baccalaureate in Mathematics, Physics and Biology with distinction. He studied at the Université Claude Bernard in Lyon, France, where he obtained in 1991 a University Degree of Technology in Applied Biology, majored in Agronomy (DUT de Biologie Appliquée, option Agronomie). He pursued his education at Rhône-Alps Agricultural Engineering School (ISARA: Institut Supérieur d'Agriculture Rhône-Alpes, Lyon, France) where he got the opportunity to visit Wageningen University (The Netherlands) in 1994 as an Erasmus student. During his stay in Wageningen he did a three-month practical period at the Laboratory of Molecular Biology (Prof. Dr. Ton Bisseling) where he worked in the Embryogenesis group (Prof. Dr. Sacco de Vries) on the genomic mapping and characterization of molecular markers involved in the embryogenesis of *Arabidopsis thaliana*. In the last year of his study at ISARA (1995), he came back to Wageningen and spent 7 months in the laboratory of Prof. Dr. de Vries to pursue his work. In the meantime he completed the program of the MSc Biotechnology, option "Plant and Microbial Production" from which he graduated in August 1995 with distinction. He graduated simultaneously from ISARA with distinction and obtained the degree of Agricultural Engineer, option "Agricultural Engineering and Environment". In December 1995, he started his military service at the Ecole Spéciale Militaire de St. Cyr, Coëtquidan, France, where he completed his officer training. Afterwards, he served as platoon commander (lieutenant) in the Groupement d'Instruction-19ème Régiment d'Artillerie, Draguignan, France, where he carried out the training of new recruits. In November 1996, he came back to Wageningen and started the PhD study described in this thesis in the Embryogenesis group (Prof. Dr. Sacco de Vries). Since November 2001, he works as a post-doc at Plant Research International, Wageningen, The Netherlands, in the laboratory of Dr. Kim Boutilier.