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Expression pattern of the *Arabidopsis thaliana AtEP3/AtchitIV* endochitinase gene

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Abstract. The carrot (*Daucus carota* L.) EP3 chitinase was shown to be essential for somatic embryo formation in a carrot mutant cell line. We identified the *Arabidopsis thaliana* (L.) Heynh. ortholog of the carrot *EP3-3* chitinase gene, designated as *AtEP3/AtchitIV* and analyzed its expression in *Arabidopsis* by means of reverse transcription-polymerase chain reaction and promoter::*β*-glucuronidase and luciferase fusions. As in carrot, the gene is expressed during somatic embryogenesis in "nursing" cells surrounding the embryos but not in embryos themselves. In plants, gene expression is found in mature pollen and growing pollen tubes until they enter the receptive synergid, but not in endosperm and integuments as in carrot. Post-embryonically, expression is found in hydathodes, stipules, root epidermis and emerging root hairs, indicating that the *Arabidopsis* chitinase may have a function that is not restricted to embryogenesis.

Key words: *Arabidopsis* (embryogenesis) – Arabinogalactan protein – Chitinase – Embryogenesis – Programmed cell death

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; Schmidt et al. 1994; Maës et al. 1997). 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 a permissive temperature (Lo Schiavo et al. 1990) while at a 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 transition from globular to heart stage, but also the formation of globular embryos (De Jong et al. 1993). Because a fully active EP3 chitinase was present in *ts11* media, 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 four members have been cloned (Kragh et al. 1996). At least five 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

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Abbreviations: AGP = arabinogalactan protein; EST = expressed sequence tag; GUS = *β*-glucuronidase; PCR = polymerase chain reaction; RFLP = restriction fragment length polymorphism; RT = reverse transcription

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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 a low level throughout the plant, but at their highest level 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. 1998a).

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 β-glucuronidase (GUS) immunolocalization, to be expressed in the aleurone, the endosperm and the embryo (Swegle et al. 1992; Leah et al. 1994), 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.

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 restriction fragment length polymorphism (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 daylight conditions, after germination on selective medium [0.46% (w/v) MS salts (Duchefa, Haarlem, The Netherlands; Murashige and Skoog 1962), 1% sucrose, 0.8% (w/v) agar, 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* mutation, 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, was constructed in the Lambda FixII vector (Stratagene) from the ecotype Ler. The library was screened with the ^{32}P-labeled tai224 full-length cDNA and five 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 polymerase chain reaction (PCR) on Ler and Col genomic DNA, using primers designed against both ends of the cDNA (TAI1: 5′-AAAATGTTGACTCCCACCATTTC-TAAATCC-3′ and TAI2: 5′-TGTTAGCAAGTGAGGTTGTTT-CCAGGATCA-3′). Sequence analysis of the different clones revealed no difference between these two ecotypes. It also revealed that the *tai224* genomic sequence was identical to that of *AtchitIV* submitted to the EMBL database by De A. Gerhardt and coworkers (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. (1998a). 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, Mass., 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 *Hin*dIII) in 1× KGB buffer [100 mM potassium glutamate, 25 mM Tris-acetate (pH 7.5), 10 mM Mg-acetate, 50 μg ml^{-1} BSA (fraction V, Sigma), 0.5 mM β-mercaptoethanol] containing 1 mM spermidine, 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. The filter was washed at 65 °C in 5× SSC (1× SSC: 150 mM NaCl, 12 mM sodium citrate, pH 7.0), 0.1% (w/v) SDS and 2× 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. 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) polyethylene glycol (PEG) 6000, 7% (w/v) SDS, 1 mM EDTA]. The probe was added and hybridization performed for 24 h at 62 °C. Following hybridization the membranes were rinsed at room temperature in 3 × SSC, 0.1% (w/v) SDS and then washed at 62 °C for 20 min in the same solution, followed by 20 min in 0.1× SSC, 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 15-kb lambda clone containing the genomic sequence of *AtEP3/AtChitIV* was used as a probe.

Analysis by RT-PCR

Reverse transcription (RT)-PCR was carried out as described by Van Hengel et al. (1998a) using 9514PP (5′-AAAATGTTGACTCCCACC-3′) as upstream primer and 9515PP (5′-TGTTAGCAAGTGAGGTTG-3′) as downstream primer for amplification of the *AtEP3/AtchitIV* reverse-transcribed cDNA. Ubiquitin was amplified as a control of the RNA using 5′-TAGAAGCTTATGCAGAT$^{C}/_{T}$TTTGTGAAGAC-3′and 5′-TATGGATCCACCACCACG$^{G}/_{A}$AGACGGAG-3′ as upstream and downstream primers respectively (Horvath et al. 1993). The 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 two-step cloning. The *AtEP3/AtchitIV* promoter was first cloned into pBluescript SK$^-$ (Stratagene) as a *Pst*I-*Xba*I 1,100-bp fragment ending directly upstream of the *AtEP3/AtchitIV* ATG, after introduction, by PCR mutagenesis, of an *Xba*I site at the 3′ end, using the primers 5′pTAI (5′-CCCTGCAGATCTTCCTGG-3′) and 3′pTAI (5′-GCTCTAGATTTGATGTTGTTGAGG-3′). Since the 5′ end of the promoter sequence already overlapped with the 3′ end of the preceding gene, we did not use a longer promoter sequence. From pBluescript the promoter was cloned in the GUS binary vector pGPTV-KAN (Becker et al. 1992) as an *Sal*I-*Xba*I fragment and as an *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 cauliflower mosaic virus (CaMV) 35 S promoter. Seeds of these plants were kindly provided by Valérie Hecht. Histochemical GUS assays were performed as described in Vroemen et al. (1996), using 2 mM 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).

Results

The Arabidopsis ortholog of the carrot EP3 genes

Searching the *Arabidopsis* genome with the carrot EP3 sequence revealed the expressed sequence tag (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 (Bacterial Artificial Chromosome) 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 (Fig. 1). 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 29.4 kDa. When compared to the EP3-3 sequence of carrot (Fig. 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 Fig. 1). The N-terminus of the mature protein then commences with a cysteine-rich region, between positions 29 and 59, which 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 Fig. 1), as are all residues assumed to be involved in catalysis (in bold in Fig. 1; Verburg et al. 1993; Andersen et al. 1997). 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 (Fig. 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 Fig. 2A), only a single protein was recognized by the antiserum in the *Arabidopsis* medium (Fig. 2A). This indicates that there might be a single ortholog of EP3 in the *Arabidopsis* genome, although seven different class-IV chitinase genes have been found so far. To demonstrate that the *AtEP3/AtchitIV* gene encodes the secreted AtEP3 protein as recognized by the carrot EP3 antiserum (Fig. 2A), the full-length *AtEP3* cDNA was intro-

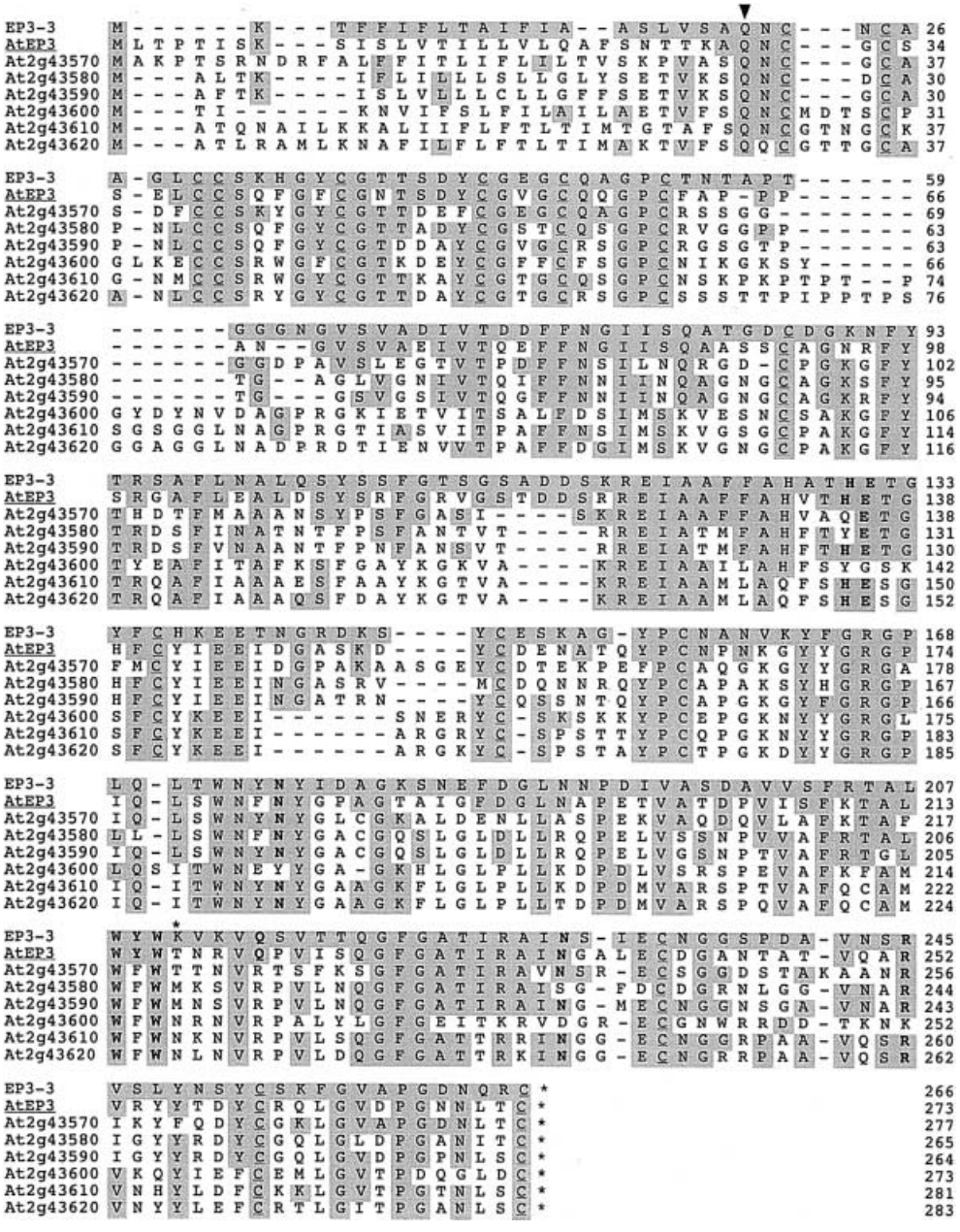


Fig. 1. Comparison of the deduced amino acid sequence of AtEP3 and other *Arabidopsis* class-IV chitinases with the carrot EP3 chitinase. Sequences are deduced from the corresponding cDNAs, EP3-3 being the carrot class-IV chitinase (De Jong et al. 1992). AtEP3 is *underlined*. Gaps were introduced for optimal alignment. The EP3-3 sequence and the residues identical to this sequence are *shaded*. The predicted signal-sequence cleavage site is indicated (*arrowhead*). Cysteine residues forming disulfide bonds are *underlined*. Residues postulated to be involved in substrate binding and catalysis are shown in *bold*. The position of a conserved methionine among chitinases of this class but absent from EP3 and AtEP3 is shown by *

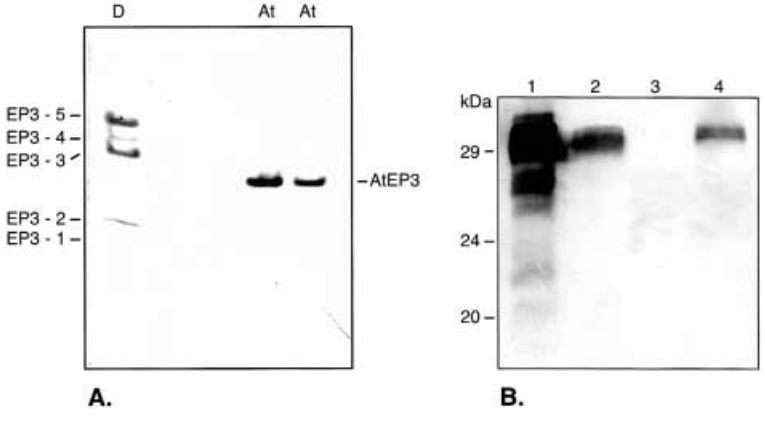


Fig. 2A,B. Immunological relationship between AtEP3/AtchitIV and the carrot EP3 chitinase. **A** Western blot of proteins from medium of *Daucus* cell-suspension cultures (*lane D*), and of *Arabidopsis* embryogenic cultures (*lanes At*), incubated with an antiserum raised against the carrot EP3 chitinase. EP3-1 to EP3-5 represent the different isoforms of the carrot chitinase and AtEP3 the corresponding *Arabidopsis* chitinase. **B** Western blot of 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)

duced 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 (Fig. 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. 1998a), AtEP3 is produced in embryogenic suspension cultures only (Fig. 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 three 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-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.

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) (Fig. 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 (Fig. 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 (Fig. 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 Fig. 3A, at the stringency used (2 × SSC at 65 °C), suggests that *AtEP3/AtchitIV* is a single-copy gene. The *Hin*dIII digestion for instance gives a single hybridizing band of 1 kb corresponding to the *Hin*dIII-*Hin*dIII fragment within the coding sequence (Fig. 3D). The completed sequencing of chromosome 3 in this region allowed the validity of the rest of the hybridization pattern to be verified, 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).

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

	Specific activity (nmol GlcNAc min^{-1} mg^{-1})	pH optimum	ts11 rescue
EP3-3 native	nd	nd	+
EP3-3 Sf21	3,200	5.0	−
AtEP3 native	6,000	4.8	+
AtEP3 Sf21	4,200	5.0	−

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 822 bp. The highest level of expression was found in seedlings (Fig. 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 used as template were comparable in each sample (Fig. 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. 1998a).

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

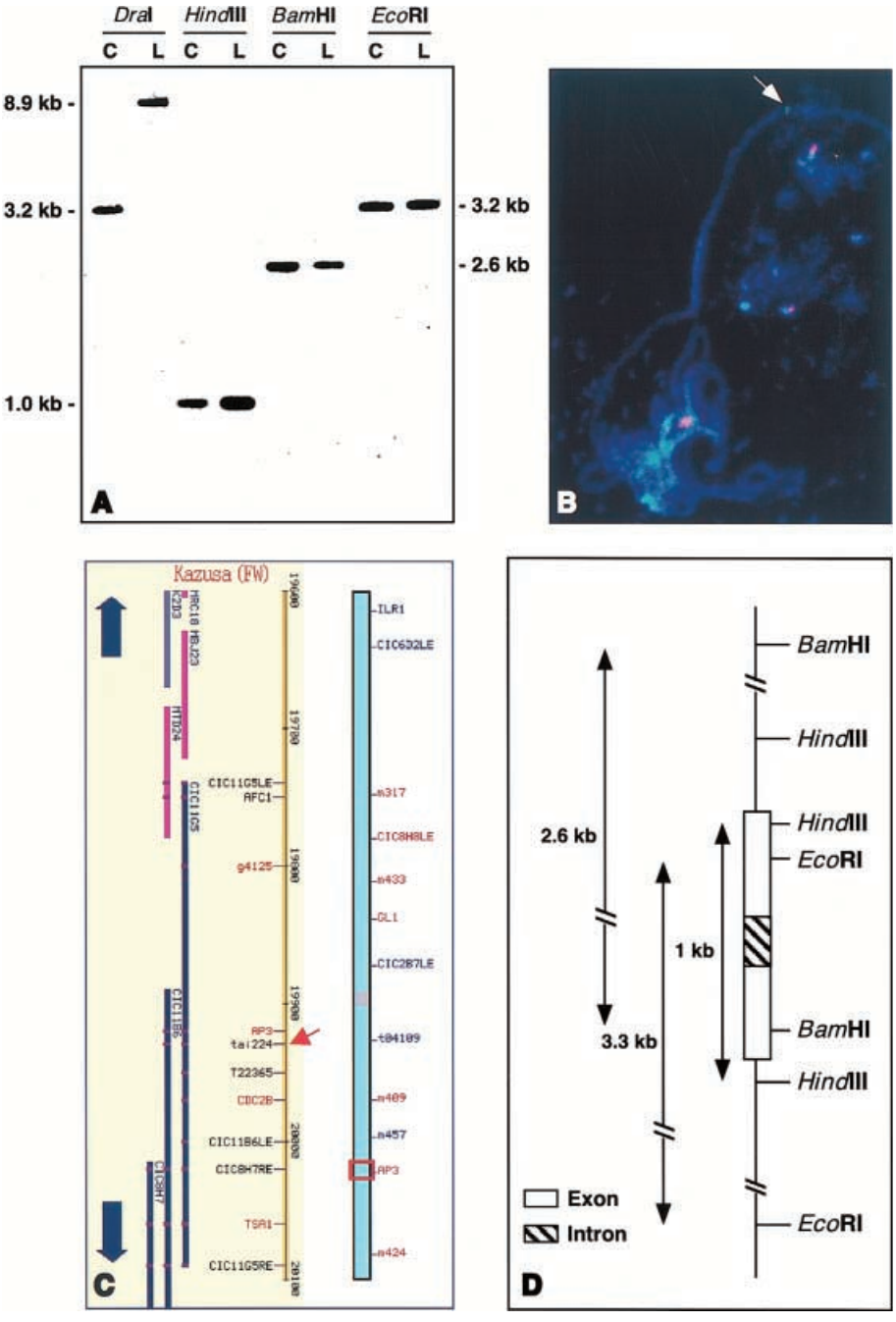


Fig. 3A–C. Genetic and physical mapping of the *AtEP3/AtchitIV* gene. **A** Genomic Southern blot from RIL parental DNA [Columbi*a* (*C*) and Landsberg *erecta* (*L*)] hybridized with the *AtEP3/AtchitIV* cDNA probe. RFLP used for the mapping was revealed by the enzyme *Dra*I (first 2 lanes). **B** Fluorescence In Situ Hybridization mapping. Superimposition of 4′,6-diamidino-2-phenylindole (DAPI)-stained pachytene chromosomes and hybridization signals. The *green signal* indicated by the *arrow* represents the *AtEP3/AtchitiIV* locus on the lower arm of chromosome 3, whereas the *red signals* represent 5 S 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

activity. No change in expression was found when compared with the GUS data (see Fig. 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. 1998a). The *AtEP3/AtchitIV* gene appeared to be expressed in cells close to the developing embryos but not in the embryos themselves. Promoter activity was observed after overnight staining in embryogenic clusters but never in the embryo at any stage (Fig. 5A). In contrast to the carrot EP3 genes (Van Hengel et al. 1998a), *AtEP3/AtchitIV* expression was restricted to embryogenic cultures and absent from yellowish non-embryogenic clusters or cultures (Fig. 5B). These results confirmed the Western blot analysis, showing that the AtEP3/AtchitIV chitinase was only detectable in the medium of embryogenic cultures (Fig. 2B).

In flowering plants, *GUS* expression was observed after 2–5 d of staining, in a stage-dependent manner in pollen. Activity of GUS was absent from young devel-

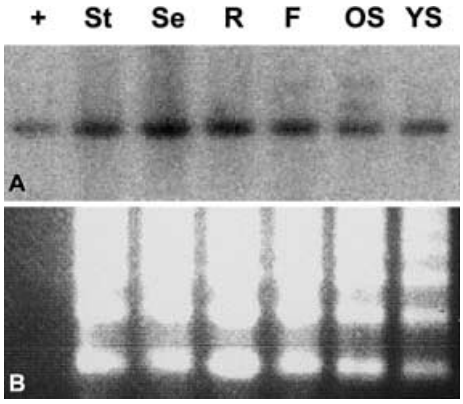


Fig. 4A,B. Analysis by RT-PCR of *AtEP3/AtchitIV* expression in *Arabidopsis* plants. The RT-PCR was performed on total RNA from stems (*St*), seedlings (*Se*), roots (*R*), flowers (*F*), old siliques (*OS*) and young siliques (*YS*). The positive control for the PCR reaction (+) was 10 pg of plasmid 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

oping pollen, and increased during pollen maturation (Fig. 6A), as was also found when measuring luciferase activity under the control of the *AtEP3/AtchitIV* promoter (Fig. 6B). β-Glucuronidase expression persisted after pollen germination in the pollen tubes growing along the placenta and the funiculus (Fig. 6C–E). Upon fertilization the GUS-expressing pollen tube entered the receptive synergid via the micropyle in order to release the two sperm cells in the embryo sac. As this occurred, both receptive synergid and central cell turned blue (Fig. 6F). While the zygote developed, GUS product remained in the degenerating receptive synergid (Fig. 6G) and in some rare cases in the free nuclear endosperm as well (Fig. 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 (Fig. 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

Fig. 6A–K. 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 stages 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 through 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

constituents of the male gametophyte including the AtEP3/AtchitIV chitinase can be transferred along with the sperm cells into the embryo sac.

Expression of GUS was not seen during embryo development up to the mature stage (Fig. 6J), except in malformed and aborted seeds, in which the embryos appeared to be misshapen (Fig. 6K). Expression of GUS re-appeared during germination in the differentiating root-hypocotyl transition zone of the young germinating seedling (Fig. 7A, B). It was also visible in some endosperm or seed coat cells at the place where the radicle protrudes (Fig. 7B, C). The cytoplasm of the cells where the staining was localized seemed to show signs of shrinkage, suggesting that these cells were dying. As the seedling developed, the transition zone differentiated, and the first root hair initials and root hairs appeared, accompanied by GUS expression in some cells of this area (Fig. 7B,D). At higher magnification, the mosaic-like staining pattern appeared to represent cells that were differentiating into root hair initials (Fig. 7E, arrowheads). Further development of the seedling confirmed the nature of this pattern (Fig. 7F,G), clearly showing GUS activity in elongating root hairs (Fig. 7G, H). Moreover the staining seemed to be confined to the epidermal layer (Fig. 7G) and extended as the specialization zone did. Staining 1-week-old seedlings for GUS showed indeed that expression remained in the root epidermis of the adult root specialization zone (Fig. 7H) and was absent from the elongation zone (Fig. 7I). In

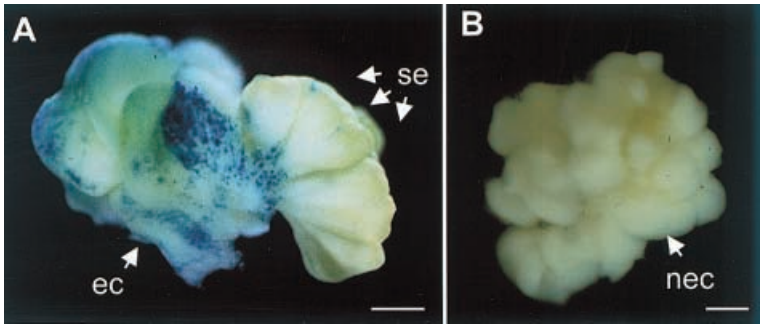
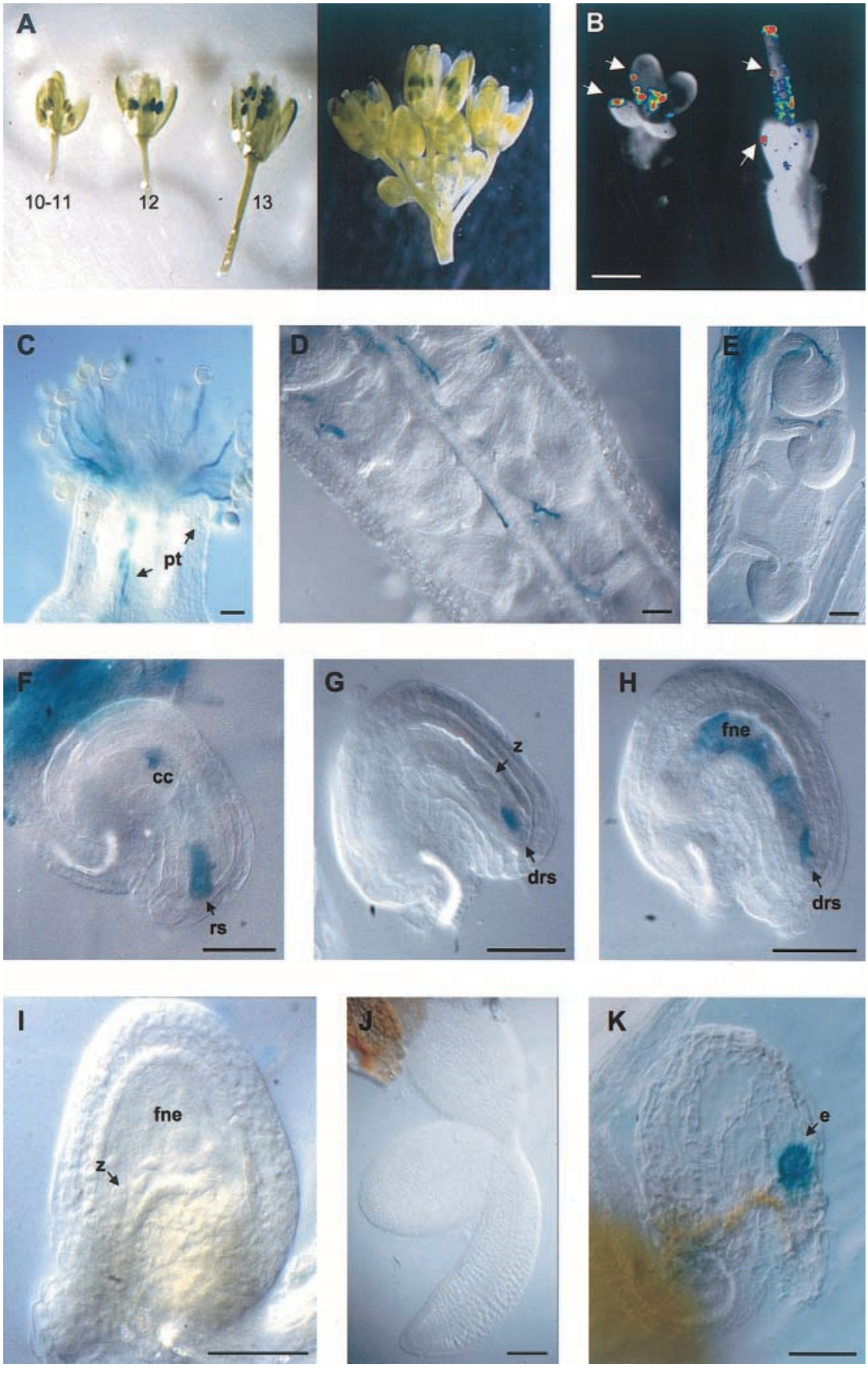


Fig. 5A,B. Histochemical localization of GUS activity during somatic embryogenesis in *Arabidopsis* cultures containing the *AtEP3/AtchitIV* promoter::GUS fusion. Embryogenic cluster (**A**) and non-embryogenic cluster (**B**) from *Arabidopsis* embryogenic lines initiated via dissection of immature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



the aerial parts of the plant, expression was restricted to the few cells forming the stipules and the hydathodes (Fig. 7H, J–L), all of which was also found in *AtEP3*/*AtchitIV::LUC* seedlings (Fig. 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. 1998a).

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.

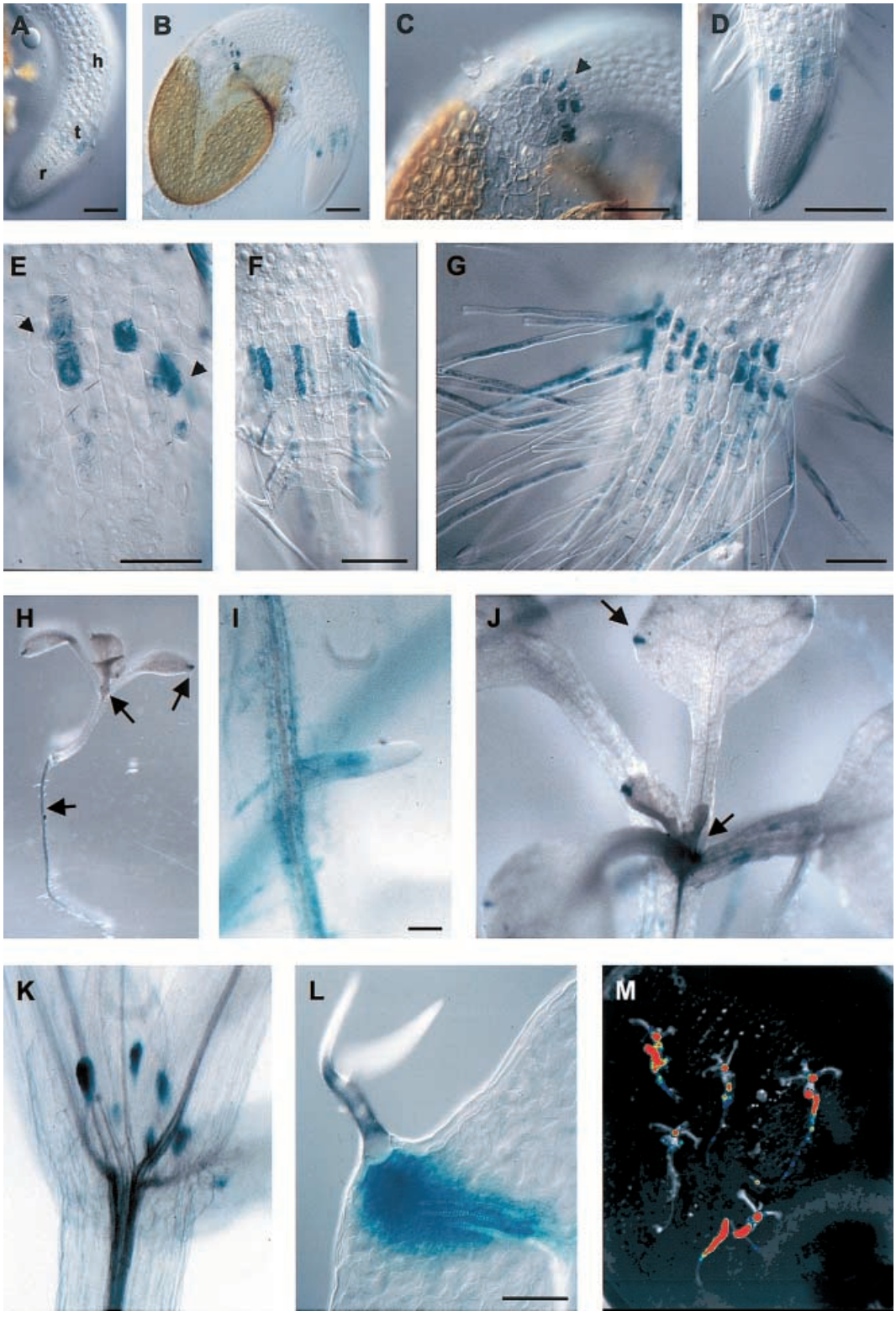
In embryogenic cell cultures of *Arabidopsis*, expression was restricted to a small subpopulation of single cells and cell clusters, and was not in the embryo itself. This confirms the hypothesis made by Van Hengel et al. (1998a) of a possible "nursing" function during embryogenesis. The *Arabidopsis* 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 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, 1993) was a reflection of their "nursing role" during zygotic embryogenesis (Van Hengel et al. 1998a). This is difficult to sustain in the case of the *Arabidopsis AtEP3/AtchitIV* gene, which is not expressed in the integuments or in the 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.

Fig. 7A–M. 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 embryonic 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** A 1-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 a hydathode expressing the GUS gene. **M** Luciferase activity measurement in 1-week-old seedlings, showing the same expression pattern as found with the GUS reporter gene. Bars = 100 μm (**A–D, I, L**), 50 μm (**E–G**)

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 arabinogalactan proteins (AGPs) in vitro (Van Hengel et al. 1998b), 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. 1998c). 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 Lind et al. 1994; Cheung et al. 1995; Du et al. 1996; Gerster et al. 1996) 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 (Schiefelbein et al.



1993; Ryan et al. 1998). Certain AGPs are also found on the root surface (Samaj et al. 1999), where they may be involved in elongation of root epidermal cells (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 expressed in the very same organs. Nevertheless, AtEP3/AtchitIV expression did not seem to change when plants were grown in sterile or challenging conditions, raising questions about a possible defense function, also suggested by De A. Gerhardt et al. (1997). Besides, many genes with unrelated func-

tions, such as a specific marker used to study the formation of the serrated margin of leaf blades in *Arabidopsis* (Tsukaya et al. 1994), *ENOD40* in *Sesbania rostrata* (Corich et al. 1998) and *fruitful* in *Arabidopsis* (Gu et al. 1998) are expressed in stipules and/or in hydathodes.

A possible function is indicated by the fact that expression of the *AtEP3/AtchitIV* gene in stipules and hydathodes corresponds with or just precedes cell death. Stipules are regarded by some (Meicenheimer et al. 1983; Medford et al. 1994) 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 by Gao and Showalter (1999) showed that perturbation of AGPs by Yariv reagent induces programmed cell death (PCD) in *Arabidopsis* suspension-cultured cells and they proposed that AGPs might be involved in other plant PCD responses as well (Dolan et al. 1995; Schindler et al. 1995; Gao and Showalter 1999). 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 transmitting-tissue-specific 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.

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