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Early elicitor induction in members of a novel multigene family coding for highly related RING-H2 proteins in *Arabidopsis thaliana*

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Abstract

We describe the identification and structural characterization of a novel family of *Arabidopsis* genes related to ATL2 which encode a variant of the RING zinc finger domain, known as RING-H2. Analysis of genes selected by us and of sequences from *Arabidopsis* stored in databases permitted the prediction of several RING-H2 proteins that contain highly homologous RING domains. The ATL gene family is represented by fifteen sequences that contain, in addition to the RING, a transmembrane domain which is located in most of them towards the N-terminal end. Transgenic *Arabidopsis* seedlings carrying the ATL2 promoter fused to the GUS reporter gene revealed that the expression of ATL2 is rapidly induced after exposure to chitin or inactivated crude cellulase preparations. Rapid induction of transcript accumulation of another member of the ATL family was also observed under the same conditions. These results suggest that some ATLs may be involved in the early stages of the defense response triggered in plants in response to pathogen attack.

Introduction

Zinc fingers are autonomously folded protein domains in which cysteine and histidine residues are used to coordinate one or more zinc ions (Berg and Shi, 1996). They have been found as part of proteins which participate in the regulation of many aspects of growth and development in eukaryotic organisms. Several classes of zinc-finger domains have been identified; most of them form part of the nucleic acid-binding domain of many proteins, often cited examples being TFIIIA, the glucocorticoid receptor, GAL4 and GATA-1 proteins (Berg and Shi, 1996; Mackay and Crossley, 1988). Zinc-finger domains are also implicated in protein/protein interactions. One example is the LIM domain that is constituted by 2 distinct zinc fingers and is found in proteins with diverse functions (Schmeichel and Beckerle, 1994). Another domain that has been suggested to be involved in protein/protein interactions is the C3HC4, also known as the RING finger (Really Interesting New Gene) (Saurin *et al.*, 1996). The RING finger motif is found in numerous proteins present in a wide range of organisms. It is encoded in regulatory proteins including viral transcription factors, oncoproteins and components of signal transduction pathways and in proteins involved in DNA repair and recombination (Saurin *et al.*, 1996). This domain differs from other zinc-binding domains in the overall primary amino acid sequence, in the arrangement of the residues which serve as ligands for zinc ions and in the tertiary structure it acquires upon folding (Saurin *et al.*, 1996).

Despite being widely distributed and present in gene products which play major roles during cell growth and differentiation, the molecular function of the RING-finger domain has not yet been well established. It has been inferred that the structure of RING may vary according to the sequences surrounding it, thereby suggesting that this zinc finger might carry out diverse molecular functions (Borden *et al.*, 1995;

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers AF132013 (ATL3), AF132014 (ATL4), AF132015 (ATL5) and AF132016 (ATL6).

Borden and Freemont, 1996). The canonical RING motif comprises seven cysteine residues and one histidine residue which coordinate the binding of two zinc ions. A variant of RING in which the metal-binding residues match the pattern of the RING motif, except for the substitution of a histidine residue for the 5th cysteine residue, has also been described. This variant, known as RING-H2, has been observed in about 10% of the proteins that harbor RING-finger domains in eukaryotes; however, functional differences between the RING and RING-H2 domains have not been established (Saurin *et al.*, 1996). Recently, various RING and RING-H2 domains have been described as part of proteins believed to be involved in ubiquitin conjugation (Kwon *et al.*, 1998; Potuschak *et al.*, 1998).

Zinc-finger genes of different types have been isolated in plants (Takatduji *et al.*, 1994; Tague and Goodman, 1995; Meissner and Michael, 1997). Among them four RING-containing proteins have been identified in *Arabidopsis thaliana*. Three of them contain canonical RING domains: COP1, a photomorphogenesis regulator that has been extensively characterized (Torii *et al.*, 1998); A-RZF, which is expressed preferentially during seed development (Zou and Taylor, 1997); and PRT1, a component of the N-end rule pathway, recently described (Potuchak *et al.*, 1998). The other is ATL2, which contains the RING-H2 variation and has been described as an early response gene (Martínez-García *et al.*, 1996). Early response genes often play pivotal roles during cell growth and differentiation (Herschman, 1991), and since ATL2 represents one of the first early response genes containing a distinct regulatory domain to be isolated in plants, we became interested in pursuing its characterization. Therefore, as part of a broad effort to understand the role of ATL2, we proceeded to isolate *ATL2*-related genes in *Arabidopsis* and to initiate the analysis of ATL2 expression.

Materials and methods

Isolation of ATL2-related genes

cDNA clones derived from *ATL3*, *4*, *5* and *6* were isolated after screening an *Arabidopsis* cDNA library in the λYES vector (Elledge *et al.*, 1991). The DNA sequence was determined from the cDNA insert of the original λYES clones. Both strands were examined by deletions generated from predicted restriction endonuclease recognition sites and by the use of synthetic oligonucleotide primers. The nucleotide sequence of ATL2 was also reexamined. A cytosine residue was found to be missing in the previously reported sequence at position 841 starting from what is presumed to be the first ATG codon. This modification changes the length of the predicted polypeptide at the carboxy-terminal end by 20 additional amino acid residues (accession number L76926). The DNA sequence of *ATL*s was determined using the DNA Sequencing Kit Sequenase version 2.0 from United States Biochemical. For this purpose single-stranded DNA was prepared for sequencing following the ssDNA template preparation procedure from Promega. The MacDNASIS ProV 3.2 and the DNASTAR programs were used for the analysis of DNA and protein sequences. Alignments were refined by hand based on the predicted amino acid sequences. Homology searches for RING-H2-related sequences were performed using BLASTP.

Nucleic acid manipulations

Standard DNA techniques were performed as described by Sambrook *et al.* (1989). Blot hybridizations were done on nylon membranes (Hybond N+, Amersham). They were carried out at 65 °C in a solution containing 0.5 M Na_2HPO_4 pH 7.2, 7% SDS, 1 mM EDTA, 1% BSA and a probe concentration of 10^6 c.p.m/ml (Church and Gilbert, 1984). Membranes were washed once at 65 °C for 20 min with a solution containing 0.1 M Na_2HPO_4 pH 7.2, 0.5% SDS, 1 mM EDTA. Two 20 min washes were then carried out with a solution containing 0.04 M Na_2HPO_4 pH 7.2, 0.5% SDS and 1 mM EDTA. For the removal of radioactive probes the following treatment was repeated at least twice: a solution of 0.5% SDS was boiled and poured over the membrane and allowed to cool to room temperature. The complete removal of the probes after each hybridization was verified by exposure of the membrane to X-ray film. For Southern blot analysis, genomic DNA was isolated from *Arabidopsis* seedlings as previously described. Then, 10 μg of DNA was digested to completiton with restriction enzymes and fractionated on a 0.8% agarose gel. The probes employed consisted of fragments containing complete cDNA inserts. For northern blot hybridization, RNA was prepared from seedlings as previously described. Samples of 20 μg of total RNA were fractionated after extraction into two identical 1.4% agarose-formaldehyde gels. The probes utilized were cDNA fragments which consisted only of the 3′-end region, and therefore were lacking in sequences

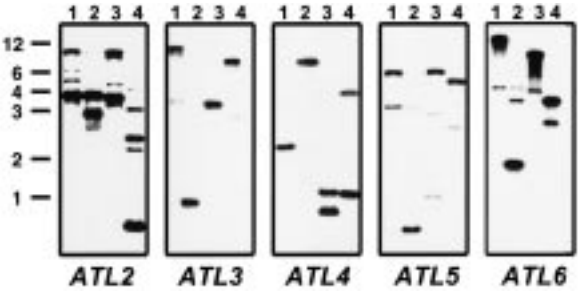

Figure 1. Southern blot analysis of *ATL* genes. 10 μg of *Arabidopsis* DNA was digested to completion with the following restriction enzymes: 1, *Bam*HI; 2, *Hin*dIII; 3, *Eco*RI; 4, *Eco*RV. The digested DNA was size-fractionated by agarose gel electrophoresis and transferred onto a nylon membrane. Blots were successively hybridized under high-stringency conditions with probes *ATL2*, *ATL3*, *ATL4*, *ATL5* and *ATL6*, as indicated; the positions of the molecular size markers (1 kb ladder from Gibco-BRL) are shown on the left.

encoding the hydrophobic regions and RING-H2 domains. The final hybridization of the northern blot membranes was performed with a probe prepared from the *Arabidopsis actin2* gene.

Plant growth conditions and generation of transgenic Arabidopsis

Arabidopsis ecotype C24 was grown in soil in a Percival Scientific growth chamber (model AR-32L). Seedlings were germinated aseptically as previously described (Guzmán and Ecker, 1990). Transformations were performed via the vacuum infiltration method (Bechtold *et al.*, 1993). Seeds of transgenic lines were germinated aseptically in plates containing MS media and 50 μg/ml kanamycin. Transgenic lines were propagated after selfing the T_3 generation of plants which were homozygous for the transgene. Three independent transformants were isolated to analyze the pattern of GUS expression. The expression pattern was very similar in all three lines (data not shown). Treatments with chitin (Sigma) and cell wall digesting enzymes, Cellulysin (Calbiochem), cellulase Onozuka R-10 (Yakult Honsha), Zymolyase 20T (Seikagaku), Lyticase (Sigma) or Novozyme 234 (InterSpex Products) were carried out in MS medium. All stock solutions were autoclaved at 121 °C for 20 min.

GUS reporter constructs and GUS expression assays

The pATL2/GUS construct carries a *Hin*dIII-*Bam*HI fragment of about 1.5 kb that includes the 5′-upstream region from the genomic sequence of ATL2; the *Bam*HI sequence is within the 7th and 8th amino acid residues of the predicted coding sequence of ATL2 (Martínez-García *et al.*, 1996). This fragment was cloned in the *Hin*dIII-*Bam*HI sites of the binary vector pBI101.2 (Clontech), generating a translational fusion.

Histochemical assays were performed essentially as described by Jefferson (1987). Briefly, young seedlings or juvenile plants were immersed in a solution containing 1 mM X-Gluc, 100 mM sodium phosphate pH 7, 0.1% Triton X-100, 10 mM EDTA and 0.5 mM potassium ferricyanide. Samples were incubated for about 16 h at 37 °C. The reaction was stopped by removing the substrate, by means of a series of three methanol/acetone (3:1) washes; samples were then placed in 50% glycerol. Tissues were analyzed under a Nikon SMZ-2t dissectin microscope. Fluorometric assay was performed as previously described (Jefferson, 1987). Protein concentrations were determined by the Bradford assay with a commercial kit (BioRad Laboratories).

Results

Identification and isolation of ATL2-related sequences

ATL2 is a recently identified early-response gene which encodes a particular variant of the RING zinc-finger domain, known as RING-H2 (Martínez-García *et al.*, 1996). RING and RING-H2 domains are ubiquitously found in eukaryotes as part of proteins involved in a wide range of processes occurring during cell growth and differentiation (Saurin *et al.*, 1996). Previous Southern blot analysis performed in *Arabidopsis* suggested the presence of *ATL2*-related sequences in the genome (Martínez-García *et al.*, 1996). To investigate the nature of such sequences we proceeded to isolate and characterize these ATL2-related clones. We screened a cDNA library of a 136 bp DNA fragment encompassing the ATL2 RING-H2 zinc-finger domain as a probe. A total of 23 positive clones were obtained and analyzed from such screening. Further analysis revealed, however, that eighteen of these clones corresponded to *ATL2*, since they hybridized to *ATL2* sequences under high-stringency conditions and they showed a similar restriction digestion pattern (data not shown). However, the sequence analysis of two of these clones indicated that they were truncated *ATL2*-encoding cDNAs. DNA sequence analysis of the other five positive clones indicated that two of these corresponded to independent cDNAs from the same gene

while each of the other three corresponded to a different gene. Sequence analysis revealed that these clones encoded previously unknown genes containing RING-H2 zinc-finger domains; we named them *ATL3*, *ATL4*, *ATL5* and *ATL6*. Southern blot analysis of *Arabidopsis* DNA performed with probes from these four genes and from *ATL2* is shown in Figure 1. For each of the probes the assay detected mostly single primary bands together with some bands of minor intensity. This indicated that the ATLs are single-copy genes which may share discrete regions of homology with other genes. We also established that the *ATL2* sequences are conserved in other plant species. We found several hybridizing DNA fragments in broccoli, pea, bean, tobacco, potato, tomato, rice and maize (data not shown), and a *Hordeum vulgare* sequence that showed strong similarity to the ATL2 was obtained from the databases (HVCH4H, see below). These observations indicate that genes closely related to *ATL2* are present in other species as well.

We took advantage of the readily available data from the *Arabidopsis thaliana* genome sequence project to estimate the abundance of RING-H2 zinc fingers in the currently available sequences from this plant. To date, about 25% of the nucleotide sequence of the *Arabidopsis* genome has been completed and annotated (Meinke *et al.*, 1998). We searched in the databases for sequences similar to the RING-H2 zinc-finger domain encoded in ATL2. At least 16 significant *Arabidopsis* matches with a RING-H2 domain resulted from the search; none of them was identical to the four new *ATL2*-related clones isolated in this work. The presence of several ATL2-related sequences in the *Arabidopsis* genome suggests that ATL2 is part of a multigene family.

ATL2 is a member of a novel family of RING-H2 zinc finger proteins

To establish any structural relationships between the various RING-H2 zinc-finger genes isolated from *Arabidopsis* in this work with those obtained from the genome sequencing projects, we performed amino acid sequence alignments between the predicted polypeptides. The alignment obtained is shown in Figure 2. Ten *Arabidopsis* sequences from genome sequencing projects and a sequence obtained from *H. vulgare* were also included in this comparison. These sequences were selected because they showed various common features when compared to ATL2 (see below). Analysis of the zinc finger region reveals a single RING-H2 domain that is very similar to the one present in ATL2. This domain displayed a high degree of identity: 28 out of the 44 residues comprising the domain are identical or showed conserved amino acid substitutions (see ATL consensus in Figure 4). This RING-H2 domain is always flanked by negatively charged amino acid residues (glutamate or aspartate) towards the N-terminal end and by positively charged residues (arginine) towards the C-terminal end. Conservation includes several other amino acids that are also found in the canonical RING domain and other residues that are conserved in most of the predicted gene products. Some of these genes may have arisen from duplication events, since in two of the cases examined they were found to be located next to each other in the genome. The predicted open reading frames F19I3.23 and F19I3.22 are located 576 nucleotides apart and T6K21.90 and T6K21.100 are 4766 nucleotides apart.

Further inspection of the predicted amino acid sequence of the sixteen ATLs revealed the presence of other domains of possible interest. Most of the predicted polypeptides include two conserved regions located between the hydrophobic region and the RING-H2 domains; one region is rich in positively charged amino acids whereas the other shows sequence conservation (Figure 2, regions marked as II and III, respectively). Towards the C-terminal end of some of the predicted polypeptides, a region of homology has also been detected (dashed outlined box in Figure 2). This region may define a subclass among ATLs. Analysis of the hydrophobic profile displayed on a Kyte & Doolittle scale is shown in Figure 3. A similar pattern of hydrophobicity is observed in most ATLs. Thirteen contain putative transmembrane domains which are at least 19 residues long, and are located at the N-terminal end; two show a hydrophobic region located toward the central portion of the protein (Z97340 and HVCH4H, in Figure 3) and one displays a long N-terminal segment with several putative transmembrane domains (T6K21.90, in Figure 3).

Relationships among closely related RING-H2 domains were also evaluated by generating a dendrogram of the predicted RING domain sequences. This analysis included closely related RING-H2 domains from *Arabidopsis* and other organisms, and canonical RING domains from *Arabidopsis*. The results obtained from the grouping of distinct sequences is shown in Figure 4. RING and RING-H2 domains divide into two distinct branches, with the canonical RING domains in one branch (Figure 4, *Arabidop-*

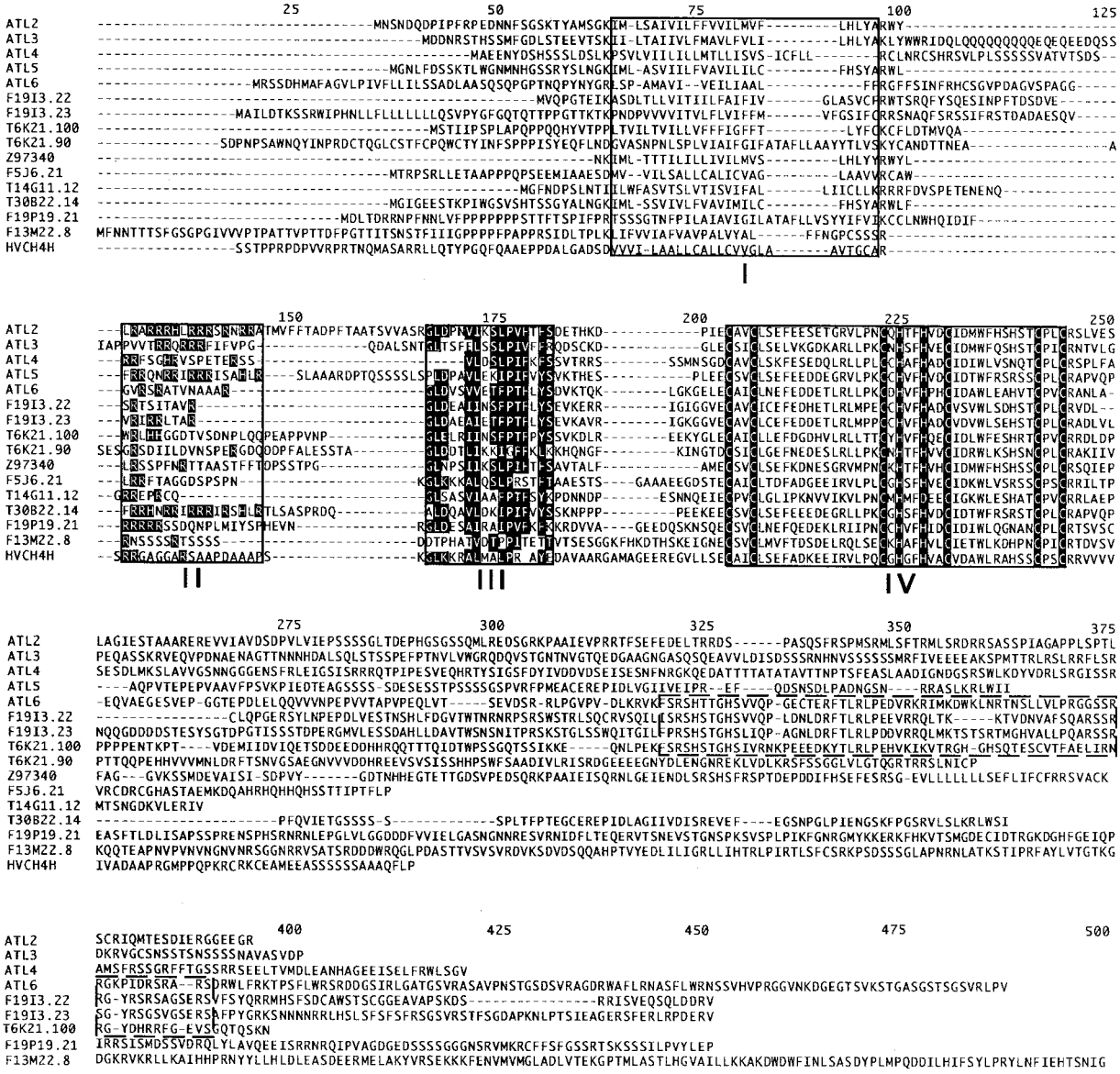

Figure 2. A family of ATL genes. The predicted amino acid sequences from *ATL* genes and from putative genes obtained from the *Arabidopsis* genome sequencing projects are displayed and aligned. Conserved regions are enclosed within boxes, I comprises the hydrophobic region which is at least 19 residues long, II comprises a basic region (basic residues are boxed in black), III represents a region conserved in most of them (conserved residues are boxed in black), and IV represents the zinc-finger domain (residues that serve as zinc ligands are boxed in black). Dashed outlined boxes identify other regions of homology. The polypeptides correspond to predicted open reading frames starting with a methionine, except for T6K21.90 and Z97340 where the open reading frames in the diagram are the conceptual translation of the exon encoding the RING-H2 domain, and HVHC4H that begins at residues 63 from the putative proteins start; other exons of T6K21.90 encode various hydrophobic regions (not shown). F13M22.8 is 630 amino acid residues long; in the diagram it ends at residue 437.

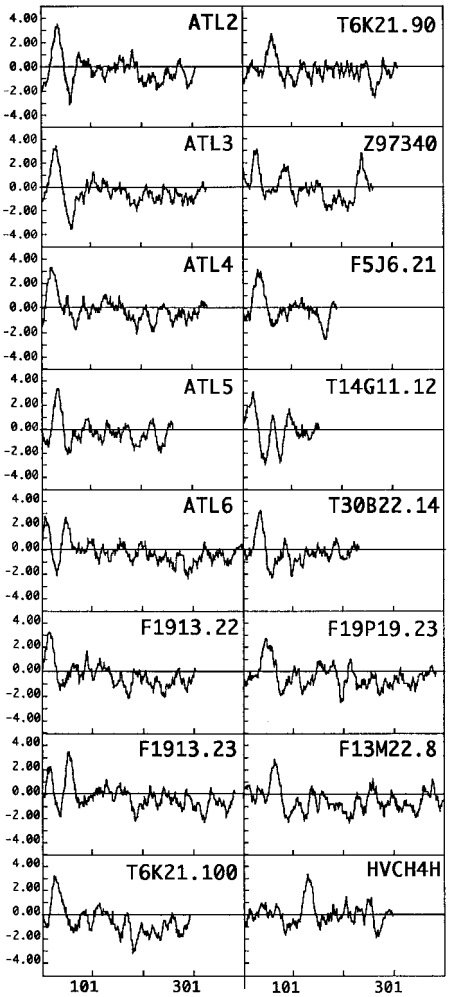

Figure 3. Prediction of the hydrophobic domain in ATL polypeptides. Plots were obtained by the MacDNASIS Pro v3.2 computer program using the method of Kyte and Doolittle with a window size of 15 residues. The ordinate indicates the hydropathic index, hydrophobic and hydrophilic values are plotted above and below respectively, and the abscissa shows the amino acid number; the predicted polypeptides are as for Figure 2.

sis canonical RING) and all the RING-H2 domains in the other. The latter further subdivide into four RING-H2 groups. The major group consists of RING-H2-containing proteins which have various common features with ATL2, and a predicted sequence from *H. vulgare* (Figure 4, ATLs). A second group is formed by plant RING-H2 domains that show different features when compared to ATLs. This group is formed by three putative polypeptides from *Arabidopsis*, one from *Glycine max*, and one from *Lotus japonicus* (Figure 4, Plant RING-H2). One of these *Arabidopsis* RING-H2 domains is encoded in a protein that contains a hydrophobic N-terminal segment with several putative transmembrane regions (F4D11.200) and the other two domains are encoded in the same polypeptide (F16B22.7 *i* and *ii*). The RING-H2 domains in the *G. max* and *L. japonicus* show a high degree of identity between them and are found close to the C-terminal end. A third group is composed by RING-H2 domains present in sequences from non-plant organisms that also include a hydrophobic region (Figure 4, Non-Plant RING-H2). These domains are found in predicted protein sequences, which have no known function. These have been identified in diverse organisms including *Schizosaccharomyces pombe*, *Drosophila melanogaster*, *Caenorhabditis elegans*, *Gallus gallus*, *Mus musculus*, *Rattus norvegicus* and man. Interestingly, three of them must be homologous genes since they showed a very high degree of identity in the overall amino acid sequence and a 100% identity in the RING-H2 domain (Figure 4, first three sequences in Non-Plant RING-H2). The fourth group is formed by the four *Arabidopsis* RING-H2 domains that contain a cluster of cysteine residues similar to a C2/C2 zinc finger motif at the N-terminal region (Figure 4, C2/RING-H2).

Pattern of expression of ATL2 in Arabidopsis *seedlings*

In order to obtain insight into the function of the ATL genes we decided to analyze the pattern of expression of ATL2 in *Arabidopsis.* Since ATL2 showed features of an early-response gene, we contemplate that the possibility that its transcription could be rapidly induced by a given stimulus would be very revealing. We constructed a chimeric gene that consisted of a translational fusion between a 1.5 kb fragment of the 5′-upstream region from the genomic sequence of ATL2 and the coding region of the GUS reporter gene. These constructs were done in the pBI101.2 binary vector (see Materials and methods), and were used to generate transgenic *Arabidopsis* plants. The expression of pATL2/GUS was examined in etiolated seedlings (5 days old), in light-grown young seedlings (5–8 days old) and in juvenile plants (15–20 days old). Low levels of GUS staining were observed in the roots of etiolated seedlings (Figure 5A), while in light-grown seedlings, GUS staining was localized to the shoot apical meristem region, with the strongest expression detected in leaf primordia and in the stipules (Figure 5B and 5C). In juvenile plants, GUS was also detected in the shoot apex region whereas

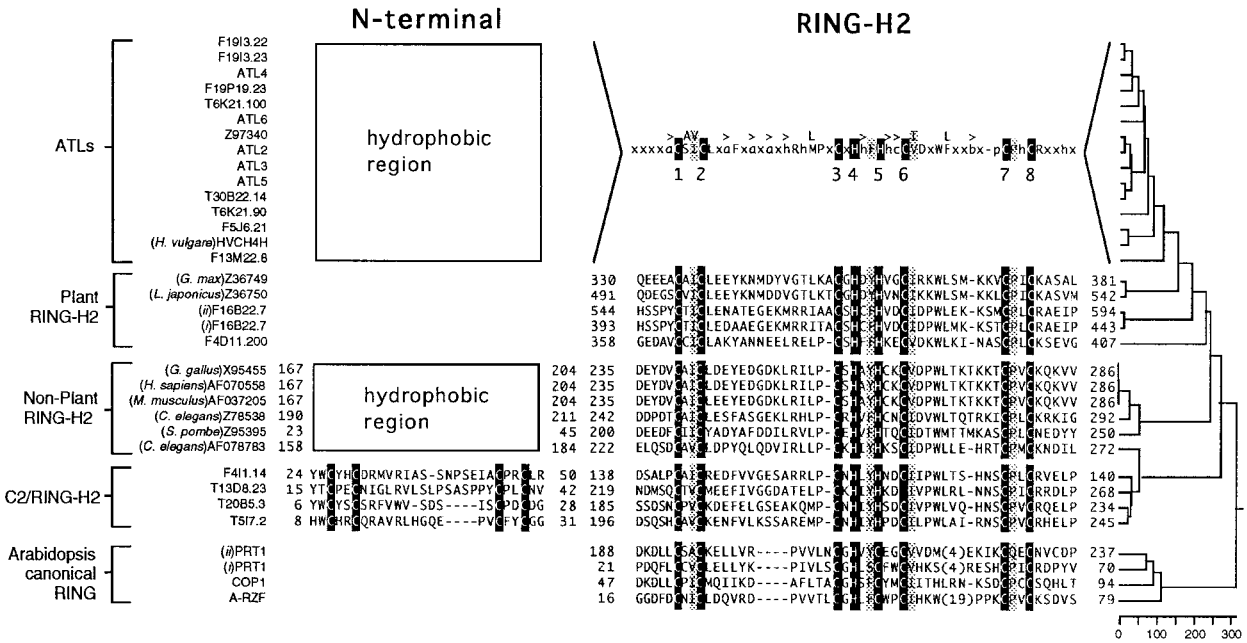

Figure 4. Sequence relationships of RING-H2 domains closely related to ATL2. To the right is the dendrogram showing the degree of relationship between closely related RING-H2 domains, performed with the Megalign program (DNAStar) by the method of Jotun Hein. Gene name or accession numbers are shown to the left of the figure. When the sequence is not from *Arabidopsis* the source organism is indicated in parenthesis. For all sequences, the translation product displaying the RING finger domain and a residue to each side of the flanking cysteine residues was analyzed; (*i*) and (*ii*) indicate each one of the two RING-H2 domains that are present in these proteins. In ATLs a consensus for the RING-H2 domain is shown, based on the alignment of the sixteen presumed proteins from Figure 2; conserved amino acid residues are denoted as follows: a, acidic; h, hydrophobic; c, charged; b, basic; p, polar; >, indicates that at least 75% of the proteins contain such residue; x corresponds to any residue. Residues believed to be involved in zinc ligation are boxed in black and numbered at the bottom of the ATL consensus, additional amino acid residues that are often conserved in RING fingers are shadowed. The boxed 'hydrophobic' represent a putative membrane spanning domain. The numbers flanking the sequences mark the position of the amino acid residues in the protein encompassing a domain. To the right, the five groups inferred from the alignment are bracketed. T14G11.12 was not used in the dendrogram since it has an aspartate residue instead of the conserved histidine residue in the central region of the domain.

low or no GUS activity was observed in expanded leaves (Figure 5D). Thus, the ATL2 promoter appears to be active preferentially around the apical meristem region, suggesting a potential role in organogenesis.

ATL2 is rapidly induced in response to potential elicitors

ATL2 displayed features often found in early-response genes. The 3′-untranslated region showed sequences which are thought to be key determinants in rapid transcript decay, and rapid accumulation of the *ATL2* transcript is observed in the absence of translation (Martínez-García *et al.*, 1996). We examined changes in the level of expression of ATL2 in *Arabidopsis* plants carrying the promoter fused to GUS. A trial designed to test the effect of regulators of plant growth and of various environmental conditions on the induction of GUS activity driven by the *ATL2* promoter was performed in young seedlings using one hour incubation periods; the results of this trial are presented in Figure 6. Previous preliminary analysis by northern blot hybridization suggested that a weak increase in mRNA accumulation was obtained after auxin treatment (Martínez-García *et al.*, 1996); this effect was reexamined and no changes in expression were observed. An increase in GUS activity was however detected when seedlings were incubated in a solution containing chitin (Figure 6). Chitin is an important component of fungal cell walls and arthropod shells. It is readily hydrolyzed into smaller fragments by the action of plant chitinases. Chitin and chitin oligomers have been used as elicitors of various defense responses in plants. Chitin elicits lignification in wounded wheat leaves (Barber and Ride, 1988), extacellular alkalinization in tomato cell cultures (Felix *et al.*, 1993), and expression of the monosaccharide H^+ symporter gene *STP4* in *Arabidopsis* (Truernit *et al.*, 1996). An increase in GUS activity is observed

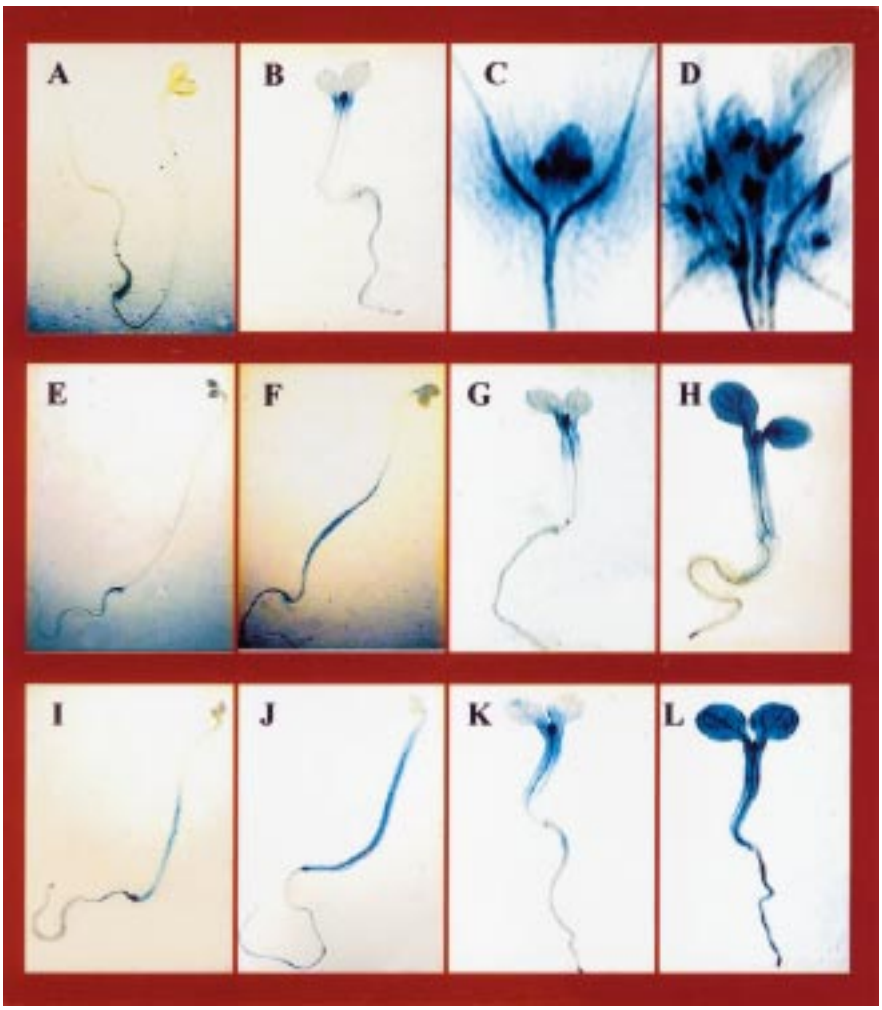

Figure 5. Detection of expression of ATL2 promoter by histochemical localization of GUS activity in *Arabidopsis* seedlings. Representative seedlings are shown. A. A 4-day old etiolated seedling. B. A 6-day old light-grown seedling. C. Apex of a 6-day old light-grown seedling. D. A 12–16-day old juvenile plant. E and F, etiolated seedling, and G and H, light-grown seedlings incubated in MS medium containing 100 μg/ml chitin for 15 min (E, G) or 120 min (F, H). I and J, etiolated seedlings and K and L, light-grown seedlings incubated in MS medium containing 100 μg/ml Cellulysin for 15 min (I, K) or 120 min (J, L).

after 15 min of incubation with chitin and continues rising after 120 min (Figure 7).

The effect of cellulases on *ATL2* expression was also tested in this assay. Cellulysin and cellulase Onozuka R-10 are crude cellulase preparations from the fungus *Trichoderma viride.* The presence of potential elicitor activity in cellulases isolated from *T. viride* has been previously reported (Barber and Ride, 1988; Threlfall and Whitehead, 1988). In some cases elicitor activity in crude cellulase preparations has been shown to be heat-stable, since inhibiting the enzyme by heating did not reduce its inducing activity (Barber and Ride, 1988). We obtained induction of GUS activity from the ATL2 promoter using autoclaved cellulase solutions, suggesting that a potential elicitor is present in these crude enzyme preparations and that its effect is not originated by the production of endogenous cell wall fragments by cellulases. A similar elicitor activity was not detected using other commercially available crude enzyme preparations of fungal origin such as Zymolyase 20T, Lyticase or Novozyme 234 (data not shown). In previously reported observations, induction of ATL2 by auxin was suggested (Martínez-García *et al.*, 1996). In these experiments, an unnoticed fungal contamination may have triggered ATL2 expression since induction of GUS activity is obtained

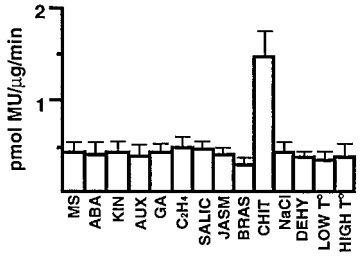

Figure 6. Search for induction of *ATL2* in young *Arabidopsis* seedlings carrying a promoter fusion to GUS. Six-day old light-grown seedlings were incubated for 1 h (except for NaCl, LOW T° and HIGH T°) in a MS medium containing: ABA, 50 μM abscisic acid; KIN, 100 μM kinetin; AUX, 50 μM 2,4-D; GA, 100 μM gibberellic acid; C_2H_4, 50 μM ACC (ACC gets readily converted into ethylene); SALIC, 0.5 mM salicylic acid; JASM, 5 μM jasmonic acid; BRASS, 50 μM 24-epibrassinolide; CHI, 200 μg/ml chitin; NaCl, 250 mM sodium cloride; DEHYD, 30 min in filter paper; LOW T°, 30 min at 4 °C; HIGH T°, 30 min at 42 °C. Fluorometric analysis of GUS activity was performed as described in Materials and methods; the result is the mean of the measurements of three different samples.

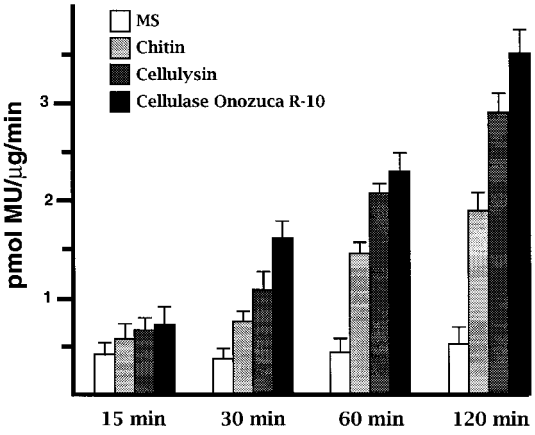

Figure 7. Induction of ATL2 promoter activity by incubation with potential elicitors in young *Arabidopsis* seedlings carrying a promoter fusion to GUS. Six-day old light-grown seedlings were incubated for the indicated time in a MS medium or MS medium containing 100 μg/ml cellulase Onozuka R10, Cellulysin or chitin; all solutions were autoclaved. Fluorometric analysis of GUS activity was performed as in Figure 6.

by the simple contact of seedlings with fungal mycelia (data not shown).

Histochemical analysis of light-grown seedlings showed that the increase in GUS activity after elicitor treatment initially appears at the shoot apical region and subsequently disseminates throughout the seedling (Figure 5G, H, K, L). In etiolated seedlings, low levels of GUS activity were initially observed followed later on by expression throughout the seedling (Figure 5E, F, I, J). Interestingly, the induction of GUS

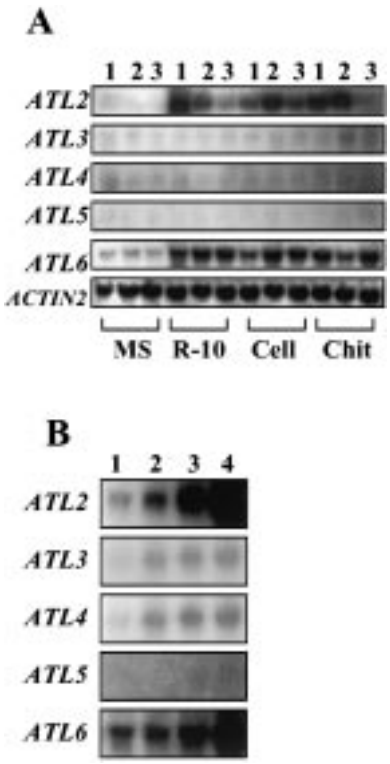

Figure 8. Effect of potential elicitors and of cycloheximide on the expression of ATL genes. Samples of 20 μg of total RNA were fractionated in two identical 1.4% formaldehyde gels and blotted onto nylon membranes. The two sets of blots were successively hybridized under high-stringency conditions with probes *ATL2*, *3* and *4* for one blot or *5* and *6* for the other; lastly, both blots were rehybridized to an *Arabidopsis* ^{32}P-labeled *ACT2* probe (only one of the blots is shown). A. RNA was prepared from 7-day old light-grown seedlings incubated in a solution containing MS medium, or MS medium with Cellulysin (Cell.), cellulase Onozuka R-10 (R10) or chitin (Chit) for the following times: 1, 15 min; 2, 30 min; 3, 60 min. B. RNA was prepared from 5-day old etiolated seedlings which were incubated in a solution containing 70 μM cycloheximide in MS medium for the following times: 1, 15 min; 2, 30 min; 3, 60 min; 4, 120 min.

from the ATL2 promoter was more pronounced when inactivated cellulases were used as elicitor (compare E–H to I–L in Figure 5, and see Figure 7).

Pattern of expression of ATL2 and ATL genes in response to potential elicitors and cycloheximide

To confirm the effect of chitin and of inactivated cellulase preparations on ATL2 expression we performed northern blot hybridization analysis. A rapid and transient increase in *ATL2* mRNA was observed in response to these elicitors: mRNA accumulation was detected within 15–30 min after incubation either with chitin or with cellulases, and then declined

(Figure 8A). mRNA accumulation of other ATL genes was also investigated by northern blot hybridization under conditions similar to those used to detect ATL2 mRNA. Induction of transcript accumulation was detected for ATL6 but not for ATL3, 4 and 5. Transcripts corresponding to these three genes were observed after extensive exposure of the autoradiogram, indicating that they have a low level of expression. The expression pattern of ATL6 mRNA differs from that of ATL2. A basal level of ATL6 transcript already present in untreated seedlings rapidly increased after incubation with chitin or with cellulases (Figure 8A). Similarly to ATL2, increase took place 15–30 min after elicitation, but, in contrast to ATL2, the levels of ATL6 mRNA did not decline after 60 min of incubation (Figure 8A). Inspection of the 3′ UTR of ATL6 did not reveal a sequence resembling a DST element which is as that predicted to be present in ATL2. It is, therefore, possible that the higher degree of stability of the ATL6 can be attributed to the absence of this particular element.

The effect of cycloheximide on ATL mRNA accumulation was also analyzed. Previous observations indicated that *ATL2* mRNA increased after incubation with this translational inhibitor. Rapid transcript accumulation after cycloheximide treatment was also detected for ATL6 but not for ATL3, 4 and 5. The pattern of accumulation is similar to that of ATL2; a rapid effect is observed and transcripts keep accumulating even after 120 min of incubation (Figure 8B).

Discussion

In this work, we describe a family of zinc finger genes in *Arabidopsis thaliana* that encode a highly related RING-H2 zinc finger domain. This family, named ATL, is represented by sixteen members: five cDNA clones isolated by this and previous efforts, ten sequences obtained from the *Arabidopsis* sequencing project and one sequence from the *H. vulgare* database. All members of this family possess a putative transmembrane domain (located in most cases at the N-terminal end) and other regions of homology. The occurrence of ATL genes in other plants was inferred from Southern blot hybridization analysis with an ATL2 probe. The first member isolated from this family shows several hybridizing DNA fragments in four plant species, and a putative gene similar to the ATLs was reported in a contiguous 60 kb sequence stretch of *H. vulgare* genomic DNA (gene name HVCH4H; Panstruga *et al.*, 1998). Although genes exhibiting RING-H2 domains similar to those present in the ATLs, together with putative transmembrane domains, can be found among predicted proteins from a wide range of organisms, these are not *bona fide* ATL homologues. This fact suggests that ATLs represent a plant-specific gene family and provides evidence that a transmembrane region associated with a RING-H2 domain may integrate a conserved functional module.

Another class of putative genes encoding similar RING-H2 zinc finger domains was also predicted from four *Arabidopsis* sequences obtained from the genome project. The four clones encode a cysteine-rich domain at the N-terminal region, $CXXCX_{(11-16)}CXXC$, which resembles a C2/C2 type of zinc finger (C2/RING-H2, Figure 4). Interestingly, the analysis of relationship performed places these predicted polypeptides in groups separated from the ATLs (Figure 4). Recently, seven tentative groups of *Arabidopsis* RING-H2 zinc finger proteins have been inferred from database searches (Jensen *et al.*, 1998). The ATL2 and the C2/RING-H2 families are among them (group E and C respectively, in Jensen *et al.*, 1998). These predictions support the fact that the RING-H2 zinc finger motif is common in plants and that the single variation in RING-H2 may have a significant role in determining the structure of the domain. The RING-H2 is a subtle variation of the canonical RING domain that is present in less than 10% of the RING fingers described to date in eukaryotes (Saurin *et al.*, 1996). It is tempting to speculate that the conservation of RING-H2 in several plant gene families, and not in other eukaryotes, took place in order to accomplish specialized functions. The imminent access to the entire genomic information from the *Arabidopsis* genome in public databases will permit a thorough analysis of the members of this multigene family (Meinke *et al.*, 1998).

Our results from northern blot hybridization are in good agreement with the expression pattern of the GUS reporter gene. Thus, a rapid increase in ATL2 mRNA accumulation after incubation with chitin or with cellulases coincides with an increase in GUS activity. When determined by northern blot hybridization, mRNA accumulation was transient, declining after 30 min of incubation. Conversely, GUS activity continued to increase after 60 min. This discrepancy is probably caused by the presence of a putative DST element in the 3′UTR of ATL2 (Martínez-García *et al.*, 1996), which would target this transcript for rapid degradation (Newman *et al.*, 1993). The pATL2/GUS

construct is more stable since it contains the 3′UTR from the *Agrobacterium tumefaciens* nopaline synthase gene. Likewise, the β-glucuronidase protein has a long half-life, and its activity does not always correlate with the levels of transcript. When the expression pattern of *ATL3*, *4*, *5* and *6* was investigated under the same conditions used to analyze *ATL2*, a transcript was observed only in the case of ATL6. Moreover, accumulation of *ATL6* was also rapidly induced after incubation with cellulases or chitin and its accumulation was still detected after a 60 min period of incubation. Its expression, therefore, appeared not to be transient, which contrasts with what was observed for *ATL2*. This difference could be attributed to the absence in the 3′UTR of ATL6 of a DST-like element similar to the one predicted in *ATL2*. Both ATL2 and ATL6 showed transcript accumulation in the presence of a protein synthesis inhibitor, which is a common feature of early-response genes. This observation indicates that the activation of these two genes is mediated by preexisting transcriptional regulators and that short-lived proteins may be negatively regulating their expression. *ATL3*, *4* and *5* are expressed at a very low level under the conditions tested, which suggests that these genes respond to other type of stimuli or that they are induced in other tissues or at different developmental stages. Low expression levels of other RING-H2 genes have also been observed (Jensen *et al.*, 1998).

The function of RING and RING-H2 domains has not been well established, although it has been suggested that they mediate protein/protein interactions and are involved in protein complex assembly. In the yeast mating response, a RING-H2 encoded in Ste5 is required to mediate association with Ste4, the β subunit of a heterotrimeric G protein. In this case, the RING domain is thought to mediate the step between G proteins and the MAPK cascade (Inouye *et al.*, 1997). In *Drosophila*, a canonical RING finger is involved in the formation of a multi-subunit complex that participates in hypertranscription on the male X chromosome (Copps *et al.*, 1998), and the yeast RING-H2 zinc finger protein Apc11p is a subunit of the anaphase-promoting complex that is required for mitotic cyclin proteolysis (Zachariae *et al.*, 1998). It has also been proposed that RING and RING-H2 domains form part of the recognition component of the N-end rule pathway in yeast, mouse, man and *Arabidopsis* (Bordallo *et al.*, 1998; Kwon *et al.*, 1998; Potuschak *et al.*, 1998; Zachariae *et al.*, 1998). The yeast Der3p, involved in endoplasmic reticulum-associated protein degradation, also possesses a RING-H2 domain; Der3p has been localized to the endoplasmic reticulum and shows a hydrophobic N-terminal region containing five putative transmembrane domains (Bordallo *et al.*, 1998). It has been hypothesized that the RING-H2 domain in this protein may participate in protein recognition for degradation (Bordallo *et al.*, 1998). The structural resemblance of ATLs and Der3p in terms of the presence of a RING-H2 domain and hydrophobic membrane spanning domains, may have functional relevance as well. Thus, some of the ATLs may be part of a protein turnover machinery. The fact that at least two of the ATLs are rapidly induced in response to elicitors known to induce a defense response in plants, allows us to speculate that these gene products may accomplish their effect by contributing to the degradation of specific proteins during the early stages of pathogen attack. Interestingly, the *Arabidopsis* disease resistance gene product RPM1 is rapidly degraded at the onset of the hypersensitive response (Boyes *et al.*, 1998). Further analysis of ATL2 and of other ATLs during plant-pathogen interaction may provide new insights into the possible role and mode of action of this novel family of zinc finger proteins.

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