The *ATL* Gene Family from *Arabidopsis thaliana* and *Oryza sativa* Comprises a Large Number of Putative Ubiquitin Ligases of the RING-H2 Type

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Abstract. Ubiquitin ligases play an important regulatory role in the control of protein degradation processes via the ubiquitin/26S proteasome pathway in eukaryotes. These enzymes participate in substrate specification and mediate the transfer of ubiquitin to target proteins. A large number of ubiquitin ligases are predicted in the eukaryotes whose genomes have been sequenced; in *Arabidopsis thaliana* more than 1300 genes are thought to encode ubiquitin ligases. At least three classes of ubiquitin ligases are present in *Arabidopsis*, one of which comprises about 470 RING zinc-finger domain proteins. Within this class we have characterized the ATL family that encodes a RING-H2 finger. We identified 80 members of this family in *A. thaliana* and 121 in *Oryza sativa*. About 60% of the rice ATLs are clustered with *A. thaliana* ATLs, and in many cases the gene products showed sequence similarities beyond the ATL's conserved features, suggesting that they could be orthologous genes. Ninety percent of the *ATLs* are intronless genes, suggesting that the structure of the basic ATL protein may have evolved as a functional module. We carried out a survey of T-DNA insertions in 30% of the *Arabidopsis ATL* genes and screened for possible phenotypes. Four of these genes are likely to be essential for viability, since homozygous plants for the T-DNA insertion were not recovered. One of them, *ATL8*, is mainly expressed in young siliques, suggesting a role during embryogenesis. We also recovered a line carrying a T-DNA insertion in *ATL43* that showed an ABA-insensitive phenotype, suggesting a role of this gene in the ABA response. The organization of ATLs in *Arabidopsis* and rice in this study will be a valuable comprehensive guide for this multigene family.

Key words: Gene families — Ubiquitin ligases — Insertional mutagenesis — ABA — Embryo lethal — Intronless genes — Defense response — Gene structure

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Introduction

The ubiquitin/26S proteasome is a primary pathway for degradation of cellular proteins that is conserved among eukaryotes (Glickman and Ciechanover 2002). This system exerts regulated degradation in multiple cellular events such as the cell cycle, signal transduction cascades, and stress responses and participates in the elimination of aberrant and truncated polypeptides. When a protein's fate is to be degraded by this system, the protein gets covalently linked to ubiquitin molecules, converting it into a substrate for degradation by the 26 proteasome. Ubiquitin conjugation occurs through a cascade of enzymatic reactions involving three classes of enzymes: E1, the ubiquitin-activating enzyme; E2, the ubiquitin-conjugating enzyme, which prepares the ubiquitin chains

to be transferred; and E3, the ubiquitin ligase that mediates the transfer of the ubiquitin to proteins and targets substrates to the proteasome—E3s are thus implicated in substrate specificity (Glickman and Ciechanover 2002).

The *Arabidopsis thaliana* genome encodes more than 1300 genes that may be involved in the ubiquitin/26 proteasome pathway. Two genes correspond to E1 enzymes, about 45 genes to E2 or E2-like proteins, and a major and diverse group of almost 1200 genes corresponds to E3 enzymes. Other elements participating in this pathway are deubiquitination enzymes and components of the 26S proteasome (Smalle and Vierstra 2004). This large number of genes involved in the ubiquitin/26S proteasome pathway indicates that the control of protein degradation has a major role in controlling cellular processes in plants. In fact, analysis of mutations in a few particular genes of this pathway has revealed that indeed it affects most cellular processes in plants (Sullivan et al. 2003; Moon et al. 2004; Smalle and Vierstra 2004).

Genes coding E3s can be classified in various groups, depending on their having the HECT domain, the RING-finger domain, or the U-box (Moon et al. 2004; Smalle and Vierstra 2004). The RING (*R*eally *I*nteresting *N*ew *G*ene) finger is a particular class of zinc-finger domain that uses a particular disposition of cysteine and histidine residues to bind two zinc ions (Freemont 1993; Joazeiro and Weissman 2000). This domain is overrepresented in *Arabidopsis*, as this superfamily contains almost 400 predicted members: 3 to 4 times more than those predicted in *Caenorhabditis elegans* or *Drosophila melanogaster* (Moon et al. 2004; Smalle and Vierstra 2004).

As in other eukaryotes, *Arabidopsis* E3s that contain a RING-finger domain function as a single subunit or as part of a multisubunit complex. As a single subunit, the RING-finger domain binds to the E2-conjugating enzyme and to the substrate; both the RING-finger and the domain mediating the recognition of the substrate are contained in the same polypeptide.

We have previously shown that some RING zinc finger genes are rapidly induced in response to elicitors. These RING fingers are members of a gene family named ATL (Salinas-Mondragon et al. 1999). The canonical RING motif comprises seven cysteine residues and one histidine residue, which coordinate the binding of two zinc ions. ATL genes contain the variant RING-H2 in which there is a substitution of a histidine residue for the fifth cysteine residue on the zinc-binding residues (Salinas-Mondragon et al. 1999). Mutants that deregulate the expression of various ATLs were isolated and their analysis revealed a link between the induction of this putative class of ubiquitin ligases and the plant defense signaling pathways (Serrano and Guzman 2004). To obtain a comprehensive guide for the ATL class of plant RING-finger genes, we have classified the ATL proteins from *Arabidopsis* and rice proteomes. The comparative analysis revealed important information about family organization and gene structure and provided hints about gene function. We found that the *Arabidopsis* family comprised 80 members and the rice family 121. The 201 classified ATL proteins form tentatively 14 groups. We analyzed potential phenotypes associated with T-DNA insertion in 24 members of the ATL family in *Arabidopsis*. The analysis of these insertional mutants showed that four *ATL* genes were essential while one more was affected in its response to ABA/glucose.
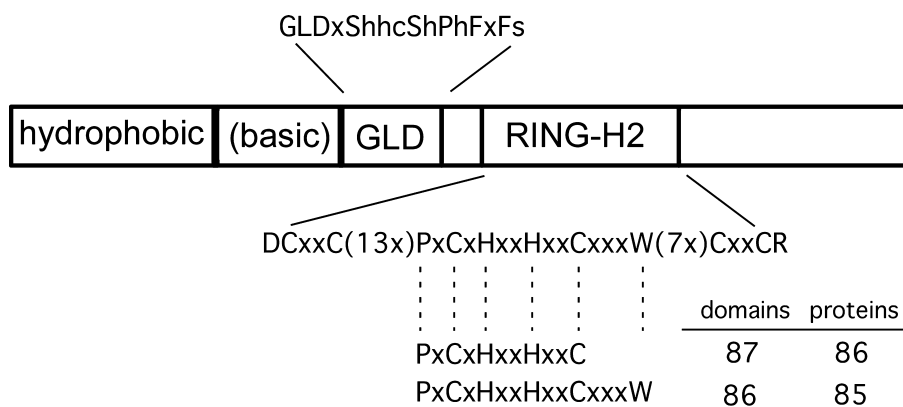
Materials and Methods

Bioinformatics. *Arabidopsis* sequences were retrieved from MIPs (http://mips.gsf.de/proj/thal/db/search/search_frame.html) and SIGnAL (http://signal.salk.edu/) databases. T-DNA insertion lines were obtained from SIGnAL http://signal.salk.edu/cgi-bin/tdnaexpress) (Alonso et al. 2003). Occurrence of cDNA clones and expression of ATL genes were inspected using the Arabidopsis Tiling Array Transcriptome Express Tool (http://signal.salk.edu/cgi-bin/atta) (Yamada et al. 2003). The pattern matching tool at TAIR (http://www.arabidopsis.org/cgi-bin/patmatch/nph-patmatch.pl) was used to search for the signatures sequences PxCxHxxHxxC and PxCxHxxHxxCxxxW in *Arabidopsis*. Rice sequences were obtained from The Institute for Genomic Research database (http://tigrblast.tigr.org/euk-blast/index.cgi?project=osa1) and from the SIGnAL (http://signal.salk.edu/cgi-bin/RiceGE) databases; occurrence of cDNA clones in rice was assessed in SIGnAL. Phylogeny reconstruction was generated using the neighbor-joining method with a bootstrap of 1000 replicates (seed = 92,738), assuming a p-distance model with homogeneous pattern of substitutions on all tested genes ($n = 201$) of *Arabidopsis* and rice; analyses were conducted using MEGA version 3.0 (Kumar et al. 2004). The multiple sequence alignment of ATL proteins was performed using Clustal X (v 1.81) (Thompson et al. 1997).

PCR Analysis of T-DNA Insertions in Arabidopsis ATL Genes. Genomic DNA was isolated from rosette leaves of plants grown in soil as previously described (Murray and Thompson 1980). PCRs were performed as follows: 35 cycles (melting, 30 s at 94°C; annealing, 1 min at 60°C; extension, 5 min at 72°C). We used a primer directed to the left border of the T-DNA: LB, 5′-GGCAATCAGCTGTTGCCCGTCTCACTGGTG-3′. Gene specific primers for *Arabidopsis* ATL genes are listed in Supplementary Table 2. PCR amplification products were resolved in 1.5% (w/v) agarose gels.

Expression Analysis by RT-PCR. RT-PCR screen was used to determine tissue specific expression of *ATL4*, *ATL6*, *ATL8*, and *ATL10* genes. Samples of total RNA from 7-day-old seedlings or selected tissues were isolated using Concert Plant RNA Reagent (Invitrogen) treated with DNAse I. Reactions were performed using Super Script One-Step RT-PCR with Platinum Taq (Invitrogen) using 100 ng RNA (DNA-free) from each sample. The thermal cycling conditions were 30 min at 50°C, 2 min at 94°C, 30–40 cycles of 30 s at 94°C, 45 s at 54°C, and 1 min at 72°C, and a

GLDxShhcShPhFxFs

| hydrophobic | (basic) | GLD | | RING-H2 | |

DCxxC(13x)PxCxHxxHxxCxxxW(7x)CxxCR

	domains	proteins
PxCxHxxHxxC	87	86
PxCxHxxHxxCxxxW	86	85

Fig. 1. Features of *Arabidopsis* ATL proteins. Schematic representation of the conserved domains in ATL proteins. The consensus amino acid sequences are based on nine ATLs. The hydrophobic region represents either single or multiple regions. The basic region is present in several ATLs but not in most of them and was not used to screen for ATLs. The pattern-matching tool at TAIR was used to search for the signatures shown below the RING-H2 sequence; the values indicate the resulting number of domains and proteins. G, glycine; L, leucine; D aspartate; S, serine; F, phenylalanine; C, cysteine; H, histidine; P, proline; W, tryptophan.

final extension of 10 min at 72°C. Amplification products were fractionated into a 1.0% agarose gel. The gene specific primers used are listed in Supplementary Table 3.

Seedling Treatments. Plants at the seedling stage were obtained after germination of surface sterilized seeds in petri dishes containing Murashige and Skoog (MS) medium (GIBCO BRL, Gaithersburg, MD) supplemented with 0.6% agar and 2% sucrose. Plates were incubated at 4°C for 4 days and then at 24°C during 7 days in a growth chamber under continuous light. Search for effects of various growth regulators was carried out at the seedling stage at the following concentrations: 10 μM gibberellic acid, 0.5 mM abscisic acid, 0.5 mM salicylic acid, 50 μM jasmonic acid, 100 μM kinetin, 50 μM 2,4-D, 0.1 and 10 μM ACC (etiolated seedlings for 72 h), and 0.1 to 3.0 μM abscisic acid; 1.0 to 7.0% glucose and mannitol were assessed in half-strengh MS medium.

GUS Expression Assays of ATL6, ATL29, and ATL36. Promoter regions of ATL6, ATL29, and ATL36 were cloned into the pBI101.2 vector (Jefferson et al. 1987). Sequence of the primers used to amplify the promoter regions are listed in Supplementary Table 4. Transformations were performed by the dip floral method (Clough and Bent 1998). Five independent homozygous T3 generation plants were propagated and the pattern of GUS expression was analyzed; the pattern was very similar in all five lines (data not shown). Histochemical and fluorometric assays were performed as previously described (Jefferson 1987).

Results

Genomewide Inspection for Arabidopsis *and Rice ATL Proteins*

We initially based our survey of *Arabidopsis* ATL proteins on a signature inferred for many ATL proteins. This signature consisted of residues located at the central region of the RING-H2 domain, two cysteines corresponding to the third and sixth zinc ligands, two histidines corresponding to the fourth and fifth zinc ligands, a highly conserved proline spaced out a residue upstream from the third zinc ligand, and a highly conserved tryptophan spaced out three residues downstream from the sixth zinc ligand (see Fig. 1). The key residue for specifically detecting ATL proteins is the proline residue. The tryptophan residue within the domain is present in many RING-finger domains as previously observed (Zheng et al. 2000). We found that almost the same number of domains were predicted in the query whether or not this tryptophan was included (see Fig. 1).

The sequences were then examined for the presence of the other modules predicted in ATL proteins (see Fig. 1). One such module is a region rich in hydrophobic amino acid residues that is located at the amino-terminal end. This module is commonly a single hydrophobic region that in most of the ATLs is at least 18 residues. However, amino-terminal ends containing two or three hydrophobic modules can also be found. A conserved motif located between the hydrophobic and the RING-H2 modules has been also predicted in ATLs. This region, named GLD (GLD denotes the first three conserved residues of the sequence), comprises about 16 residues where a glycine and a proline residues are highly conserved and the distance between them is almost invariable. Although a region rich in basic residues has also been predicted in various ATLs, these basic modules were not noticeable in most of them, and thus it was not considered a requirement for classification in our search for ATL members.

We confirmed and strengthened our results by comparing our survey to a previous computational analysis of RING fingers in *Arabidopsis* (Kosarev et al. 2002). In that study, 387 RING domains were predicted and grouped. Three main classes, corre-
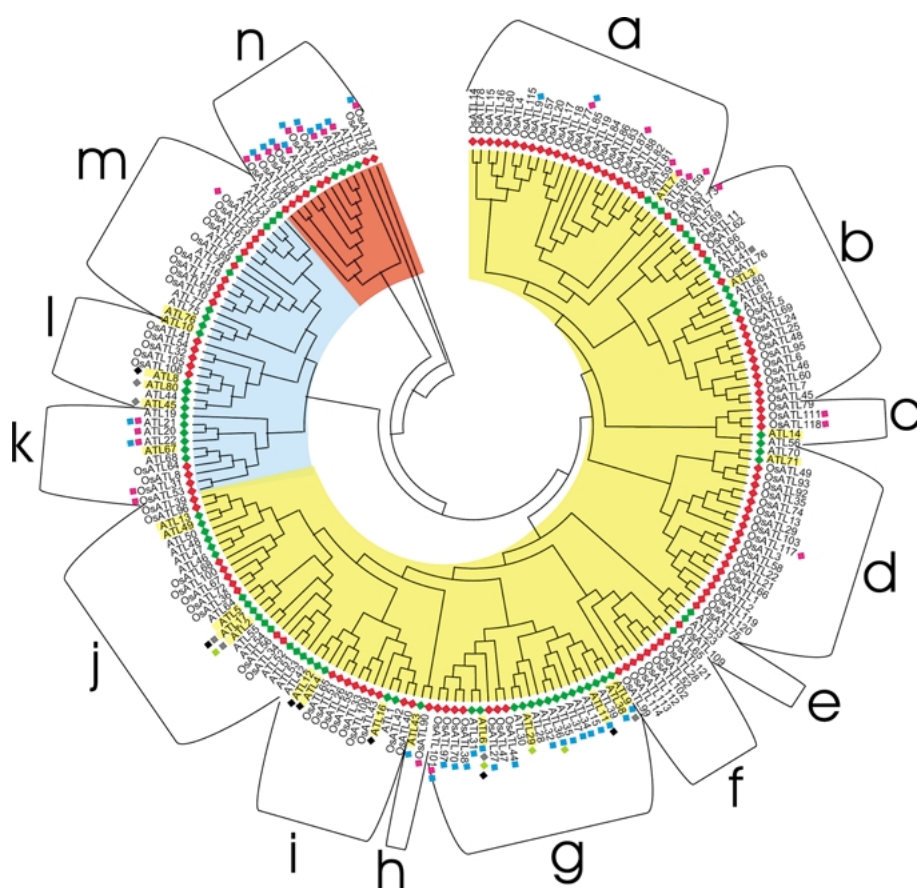
Fig. 2. Phylogeny of *Arabidopsis* and rice ATL proteins. The neighbor-joining method was used to construct the tree (see Materials and Methods and Supplementary Information). Each one of the three major clades is shown in a different color. Green and red squares highlight *Arabidopsis* and rice ATLs, respectively; the corresponding genes containing T-DNA insertions analyzed are highlighted in yellow. The branches were assembled in 14 groups, a to n, based on a protein distance matrix. Pink squares point out proteins with more than one exon; blue squares, proteins with more than one hydrophobic region toward the amino end; gray squares represent genes induced in response to flagelin; black squares indicate genes induced in *eca* mutants; and light green shows promoter fusions that are induced by elicitor.

sponding to the canonical RING, to the RING-H2, and to RING variants, were described. The RING-H2 class was found to consist of 215 domains in 214 proteins. One of the clusters (cluster 2.1) groups 75 proteins that include most of the initially described ATLs (Kosarev et al. 2002). The ATL proteins identified in this work correspond to this cluster plus two members from another cluster (Supplementary Table 1). A few other differences were detected, such as new genes or changes in gene names (see Supplementary Table 1). At the end of our analysis, 80 *Arabidopsis* ATL proteins were predicted. This number is also consistent with the results from another analysis of *Arabidopsis* RING zinc fingers that predicted 79 RING-H2 proteins containing a hydrophobic domain (Greve et al. 2003).

A detailed draft of 95% of the rice genome is currently available for analysis. To identify ATLs in the rice proteome, reiterative searches for the RING-H2 motif of ATL2 and ATL6 on the rice protein sequences databases were performed using BLAST. The sequences were then inspected for the presence of both the hydrophobic and the GLD modules; 121 rice proteins that contained these features were predicted. Availability of cDNA clones and transcript verification by whole-genome array showed that 70 of the *Arabidopsis ATL* genes are expressed; likewise cDNA

or EST clones have been isolated from 62 of the rice *ATL*s (see Supplementary Table 1).

Relationships Between ATL Proteins

Analysis of the phylogenetic tree containing 201 ATL protein sequences revealed three distinct clades; these clades were assembled in 14 groups based on a protein distance matrix (Fig. 2 and Supplementary Fig. 1 and Table 1). Clade 1 (Fig. 2; shadowed in yellow) contained 75% of the protein sequences, of which a great number belonged to rice. The other two clades contained a similar number of *Arabidopsis* and rice protein sequences (Fig. 2; shadowed in blue and red). Defined groups of presumable paralogues are readily identified, Rice paralogues were contained in groups a, b, d, and f, and *Arabidopsis* paralogues in groups g, k, l, and m. Likewise, several of the groups of the *Arabidopsis* and rice predicted proteins had a wide distribution across the dendrogram with comparable numbers between clusters, suggesting that similar selective pressure has driven the growth of the ATL family by means of internal duplications. For instance, clade 3 (Fig. 2; shadowed in red) included mostly proteins encoded by genes containing introns, a feature not shared by most *ATLs* (see below and Fig. 5).
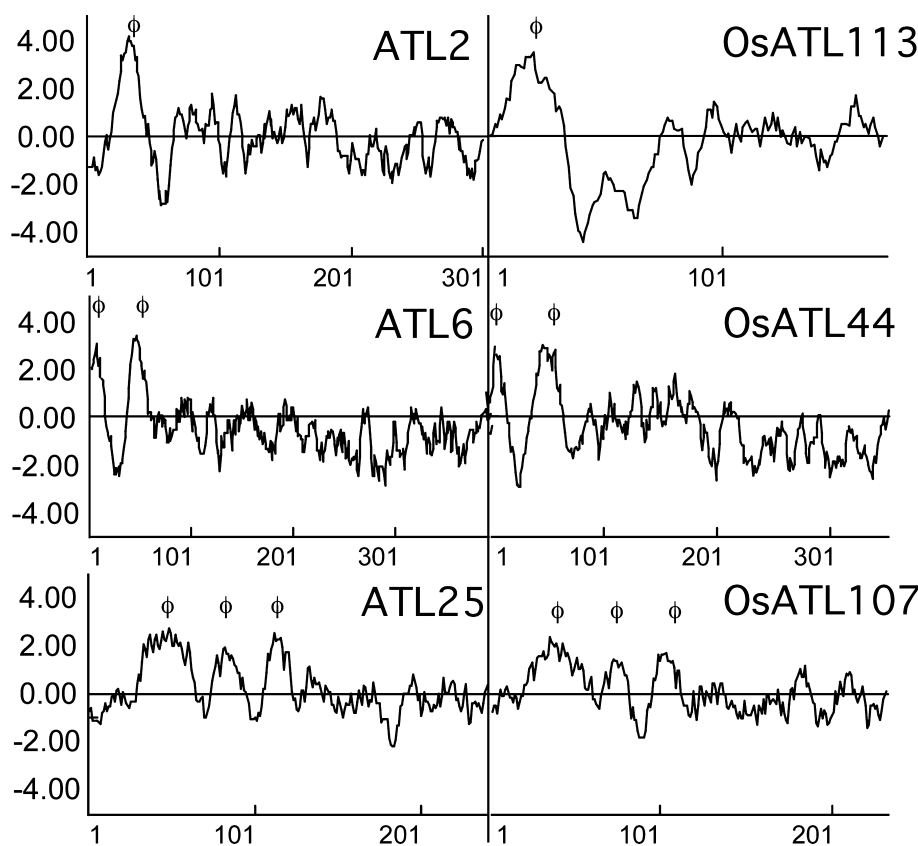
Fig. 3. Prediction of the hydrophobic domain in ATL proteins. Diagrams were created by the MacDNASIS Pro v3.5 program using the method of Kyte and Doolittle with a window size of 15. In each diagram, the ordinate displays hydrophobic and hydrophilic values, plotted above and below, respectively. The abscissa shows the amino acid number; φ represents a hydrophobic segment of the protein.

Features of ATL Genes

A distinct feature of the ATL family is the presence of a hydrophobic domain located at the amino-terminal end that typically spans over 15 amino acid residues (Salinas-Mondragon et al. 1999). Most of the ATLs include a single hydrophobic region (Fig. 3; upper panel), but we found that two and up to three hydrophobic regions can also be predicted in some members (see Supplementary Table 1). Two hydrophobic regions are common in proteins from group g (Fig. 3; middle panel), while three hydrophobic regions are evident in group n (Fig. 3; lower panel).

The position of the 80 *Arabidopsis ATL* genes on the five *Arabidopsis* chromosomes is shown in Fig. 4. Six tandem duplications of genes were detected; four corresponded to sets of two genes, and two to sets of four genes. Except for the ATL29/ATL53 set from chromosome 4, proteins from each set were grouped within the same cluster, indicating that they may have arisen from recent gene duplication events. Similarly, about seven tandem duplications of *OsATLs* were also detecting (data not shown), supporting the fact that various *ATL* genes are formed by means of linear-specific expansion.

Gene structure analysis showed that almost 90% of the *ATLs* were intronless genes (70 of 80 *Arabidopsis ATLs* lack introns and 102 of 121 rice *ATLs* lack introns; see Supplementary Table 1). Eleven of the intron-containing *ATLs* were clustered in a separate clade (clade 3 or group n in Fig. 2). With the exception of *OsATL98*, the members of this group contain four introns that separate distinct domains. The first three exons encode regions rich in hydrophobic residues, the fourth exon includes the conserved region and the first pair of cysteine residues from the RING-H2 domain, and the fifth exon includes the rest of the RING-H2 domain. *OsATL98* lacks the second and third introns and instead includes the GLD region and the RING domain in a single exon (see Fig. 5; group n).

Additionally, five sets of two to four genes containing introns were randomly distributed among the groups; three of them are depicted in Fig. 5. *Arabidopsis* genes clustered within group a contained two introns. In this case, each of the three modules, the hydrophobic region, GLD region, and RING-H2 finger, is encoded in a different exon. Groups c and k each comprise two rice genes in which two or three modules are also separated by introns (Fig. 5).

Survey of T-DNA Insertions in ATL Genes

The function of *ATL* genes in plants has not yet been established, although several of them catalyze polyubiquitination in vitro (Takai et al. 2002; Stone et al. 2005). To gain insight into the role of members of this
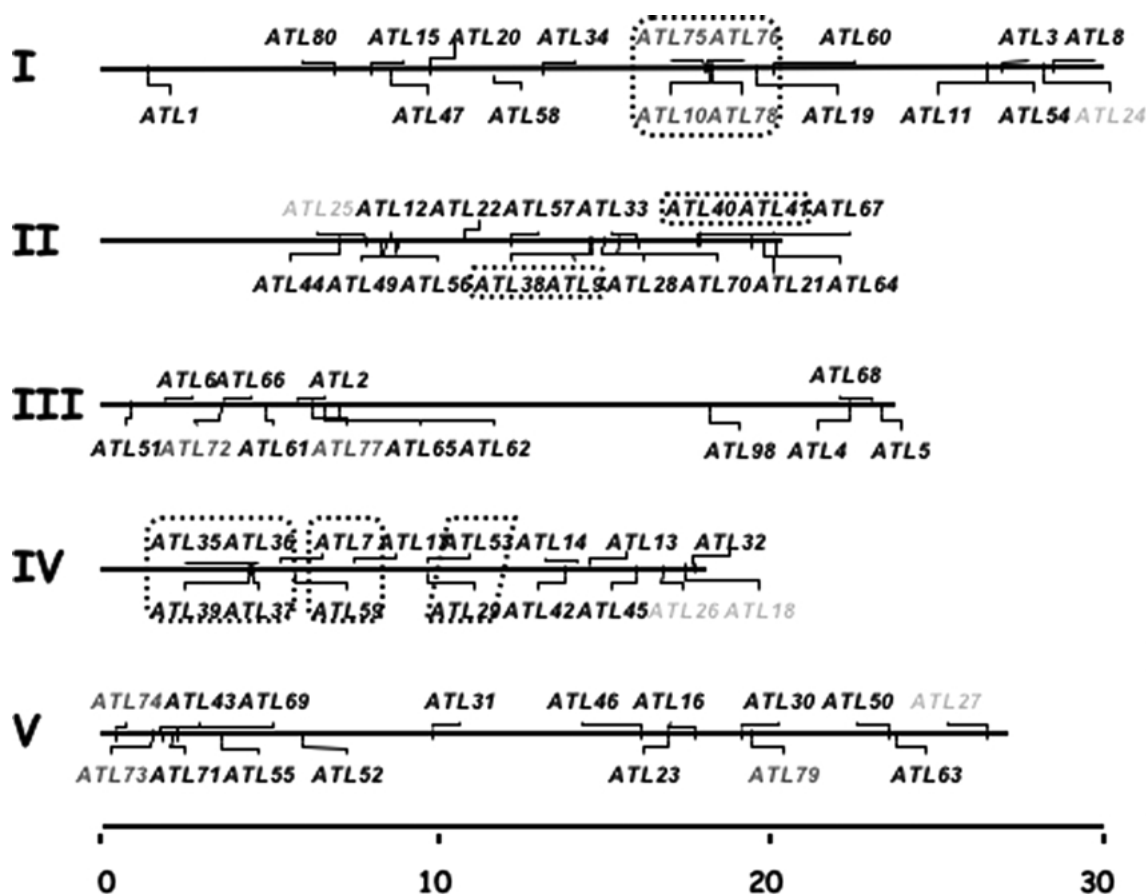
Fig. 4. Distribution of *ATL* genes on the five *Arabidopsis* chromosomes. The chromosomes of *Arabidopsis* are indicated and schematically represented by lines. The relative position of the 80 *Arabidopsis ATL* genes is indicated. Regions containing possible gene duplications are enclosed in dashed squares. Genes from clade 1 are indicated in black, those from clade 2 in gray, and genes from clade 3 in light-gray.

family and to have an initial clue about the degree of functional redundancy in this family, we looked for phenotypes of *Arabidopsis* lines carrying mutations in *ATL* genes. Since loss-of-function mutations in RING-finger genes of the ATL family have not arisen from genetic screens described in the literature, we analyzed the phenotypic consequences of T-DNA insertional mutations in *ATL* genes. T-DNA insertion mutant lines for 24 *ATL* genes were obtained from the SALK sequence-indexed insertion mutant database (Alonso et al. 2003) (see Supplementary Fig. 3); lines were genotyped to identify homozygous lines for the corresponding T-DNA insertion.

Homozygous plants carrying T-DNA insertions were obtained for 20 of the *ATL* genes, and in 4 cases only hemizygous lines were recovered. The homozygous lines were examined under standard growth conditions; after visual inspection of the plants no morphological, growth, or developmental alterations were detected in any of these mutant lines (data not shown). An assortment of conditions was then assayed to try to find phenotypes in such mutant lines. We tested for the effect of regulators of plant growth and of various environmental conditions on the

growth of young seedlings (see Materials and Methods). In this survey, one mutant line exhibited an altered response; a T-DNA insertion in *ATL43* shows insensitivity to abscisic acid (see below).

We reasoned that a cause for the lack of detectable phenotypes might be the occurrence of gene redundancy. With the support of the phylogenetic tree we investigated possible structural similarities between ATLs. We mainly based our analysis on comparing the protein sequences downstream from the RING-H2 sequence. In most cases, one or a few proteins similar in sequence were observed (see alignments for ATL7, ATL3, ATL71, ATL16, ATL12, ATL5, ATL13, ATL49, ATL67, ATL80, ATL76, and ATL45 in Supplementary Fig. 2). Likewise, from a branch of 13 *Arabidopsis* proteins, 4 mutant lines did not show a readily detectable phenotype (ATL11, ATL9, ATL38, and ATL29, from a branch in group g) (see Supplementary Fig. 2e). These observations suggest that structurally related genes exist for most ATLs. When similarities were not detected toward the carboxy end, regions flanking the hydrophobic domain were analyzed (see ATL14 in Supplementary Fig. 2c). ATL43 got sep-
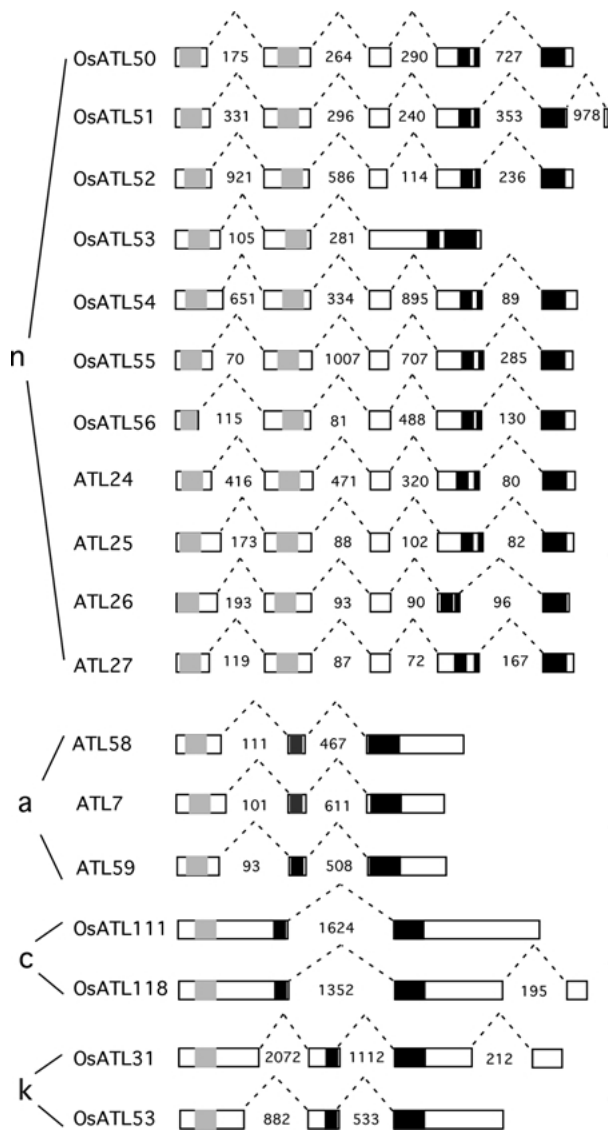
Fig. 5. Intron-exon organization in three groups of *ATL* genes. Exons (boxes) and introns (dashed lines) are shown. The length of introns in base pairs is indicated below the intron. Light-gray boxes represent hydrophobic regions, dark-gray boxes the GLD region, and black boxes the RING-H2 domain. The letter code to the left indicates the group to which it belongs (see Fig. 2).

arated with a rice ATL in a unique branch. A T-DNA insertion in this gene results in insensitivity to abscisic acid; in this case functional redundancy may be discarded.

Four Lines Carrying T-DNA Insertions in Essential Genes

In four cases, only hemizygous lines were recovered, suggesting that the homozygous lines for the T-DNA insertion were not viable. This observation was further confirmed by analyzing the segregating progeny of a hemizygous line. T-DNA insertions analysis in *ATL4*, *ATL6*, *ATL8*, and *ATL10* yielded lines lacking the corresponding T-DNA and lines harboring hemizygous T-DNA insertions but not lines homozygous for the T-DNA insertions; the observed segregation ratio is compatible with the expected 1:2 ratio between wild-type and hemizygous plants (see Table 1). A seedling-lethal phenotype was not detected in either of the mutant lines, suggesting that the mutation may be affecting embryogenesis or gamete formation (data not shown).

ATL4 is in the same branch with ATL12 and ATL42, but it showed fewer similarities to these two proteins, suggesting that there are no proteins closely related to ATL4 (see Fig. 2 and Supplementary Fig. 2i). A structural difference was detected in ATL10. The predicted sequence for ATL10 showed three copies of a 13-amino acid residue sequence that is present only once in the other three proteins of the same branch (Supplementary Fig. 2n). The other two ATLs had structurally related proteins. ATL6 showed similarities to ATL31 which were included in a branch on group g and ATL8 showed similarities to ATL80 (Supplementary Figs. 2f and m, respectively).

We evaluated the expression of these four *ATL* genes in different tissues. RNA was extracted from seedlings, roots, rosette leaves, stems, cauline leaves, inflorescences, and immature siliques and expression was assessed by RT-PCR (Fig. 6). Transcripts corresponding to *ATL4* and *ATL6* were detected in all tissues tested, except for roots. It was shown previously that *ATL6* is induced by elicitors, whereas *ATL4* is not (Salinas-Mondragon et al. 1999). For *ATL8*, expression was mainly observed in inflorescences and immature siliques, suggesting that it may be specific for reproductive tissue. Expression of *ATL10* was not detected in any tissue, indicating that its expression may be restricted to certain conditions not used by us, since there are cDNAs reported for this gene (see Supplementary Table 1).

A Line Carrying a T-DNA Insertion in ATL43 *Shows Insensitivity to Abscisic Acid (ABA)*

A line carrying a T-DNA insertion in *ATL43* showed a distinct phenotype: when germinated in ABA-containing media, it displayed an ABA-insensitive phenotype. The progeny of 10 individual plants was tested again for the phenotype and in all cases seedlings displayed the ABA-insensitive phenotype. A further analysis of this phenotype indicated that it was intermediate compared to the ABA-insensitive mutant *abi4* (Fig. 7a). The *abi4* mutant germinates in high levels of ABA as well as in high levels of glucose (Leon and Sheen 2003). Since crosstalk between the ABA and the glucose pathways occurs in plants, we

Table 1. Segregation analysis of insertion lines in essential *ATL* genes

|	Wild type (no T-DNA)	Hemizygous (one copy)	Homozygous (two copies)	χ^2
ATL4	12	34	None	1.0894; $p < 0.05$
ATL6	15	33	None	0.0937; $p < 0.05$
ATL8	12	35	None	1.2812; $p < 0.05$
ATL10	13	36	None	1.0226; $p < 0.05$
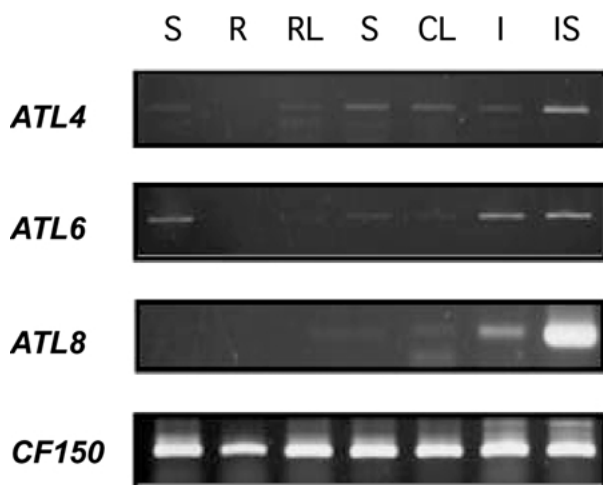
Fig. 6. Expression pattern of essential *ATL* genes in *Arabidopsis*. RT-PCR (reverse transcription with PCR) assays were performed from total RNA isolated from seedlings (S), root (R), rosette leaves (RL), stems (S), cauline leaves (CL), primary inflorescences (I), and immature siliques (IS). One hundred nanograms of DNAse I-treated RNA was used for each reaction and the resulting products were fractionated in a 1.0% agarose gel. Sizes of the RT-PCR products are as follows: *ATL4*, 1005 bp; *ATL6*, 1197 bp; *ATL8*, 398 bp; and *ATL10*, 310 bp. *CF150* (At1g72150) was used as a constitutive expression control. Under the conditions used no amplification was detected for *ATL10* (data not shown).

tested for the effect of glucose in the *atl43* mutant (Leon and Sheen 2003). *atl43* showed an intermediate glucose-insensitivity phenotype compared to *abi4* (Fig. 7b); no effect was observed when mannose medium was used as a control (Fig. 7c).

## ATL Genes Participating in the Elicitor Response

We previously showed that *ATL2* is induced by chitin or cellulysin and that it exhibits a distinct expression pattern after incubation with these elicitors of the defense response. Likewise, by Northern blot analysis we detected induction of *ATL6* in response to the same stimuli. ATL6 belongs to group g, most of whose members encode two hydrophobic regions at the amino terminus instead of one and a distinct carboxy-terminal region. We therefore tested *ATL6* and two other members of this group for chitin or cellulysin iduction. Expression of GUS fusions to promoters of *ATL6*, *ATL29*, and *ATL36* was examined in light-grown young seedlings (Fig. 8). *ATL29*
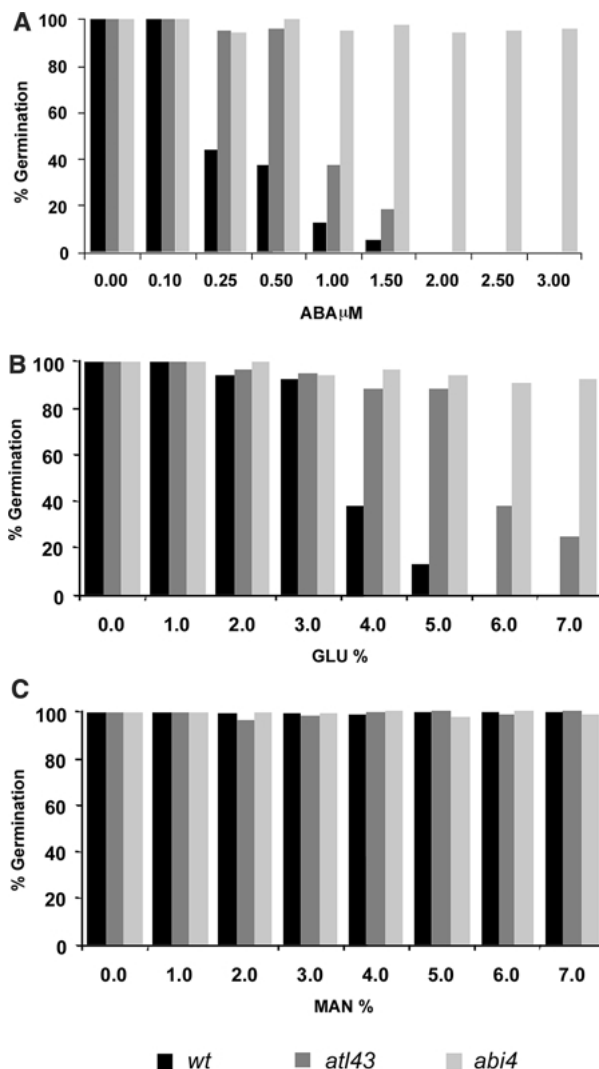
Fig. 7. Inhibition of seed germination in *Arabidopsis atl43* by ABA and glucose. Vernalized seeds from wt, *atl43*, and *abi4* were germinated for 7 days in MS media containing increasing concentrations of ABA (**A**), glucose (**B**), and mannitol (**C**). Germination percentage is indicated as the number of seeds showing cotyledon emergence. Data are means of a single duplicated experiment.

and *ATL36* displayed localized GUS staining to the shoot apical meristem region, a pattern similar to that previously reported for *ATL2*. *ATL6* showed a higher basal expression throughout the cotyledonary leaves. An increase in GUS activity was detected in the three plants carrying the GUS constructs after incubation in a solution containing elicitor. Histochemical
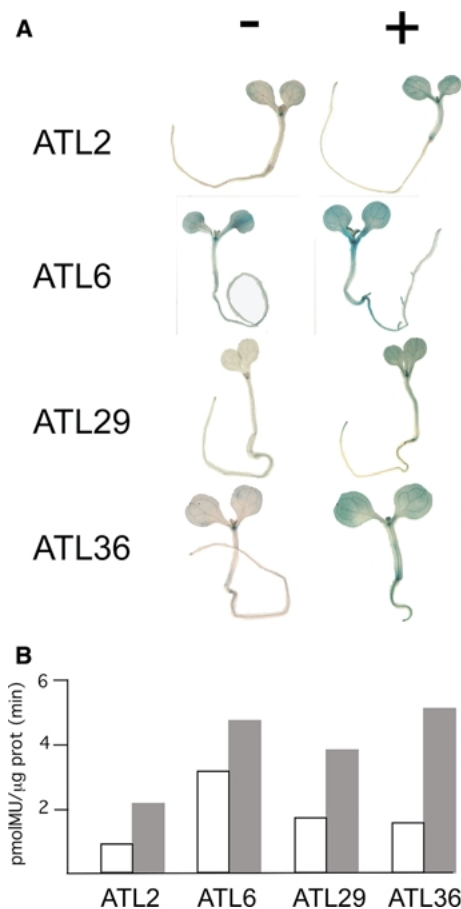
Fig. 8. Induction of *ATL2*, *ATL6*, *ATL29*, and *ATL36* promoter activity by incubation with cellulysin in young *Arabidopsis* seedlings carrying promoter fusions to GUS. **A** Histochemical localization of GUS activity in 6-day-old light-grown *Arabidopsis* seedlings incubated in MS medium containing 100 mg/ml cellulysin for 120 min; representative seedlings are shown. **B** Fluorometric analysis of GUS activity was performed as described under Materials and Methods; the result is the mean of the measurements of two different samples.

analysis showed that GUS activity after elicitor treatment disseminates throughout the seedling as previously seen for *ATL2*. These observations indicate that structurally related *ATL* genes may be involved in the defense response, as previously inferred (Salinas-Mondragon et al. 1999; Serrano and Guzman 2004).

Discussion

To develop a comprehensive guide for the ATL family, a multigenic family of putative ubiquitin ligases, we identified the predicted *ATL* genes from *Arabidopsis* and rice. The ATL family is an important class of RING-H2-finger genes since it represents about 40% of the RING-H2-finger genes in *Arabidopsis*. Our study detected 201 ATL proteins, 80 from *Arabidopsis* and 121 from rice. Inspection of databases showed evidence of expression for about 90% of *Arabidopsis* ATLs and 50% of rice ATLs (the reduced number for rice may only be a sign of the less extensive analysis of the rice transcriptome). This observation suggests that most *ATL*s are active gene forms rather than pseudogenes. Although the function of this family of ubiquitin ligases is virtually unknown, we show initial evidence of the involvement of some of its members in the growth-regulator response, response to biotic stress, and plant development. These results support the suggestion that a large number of members of this multigene family participate in the regulation of plant-specific processes. Selective constraints and the need for divergence within this gene family to respond to a wider number and variable stimuli may have driven its expansion.

About 60% of the rice ATLs are clustered with *Arabidopsis* ATLs, and for many of them their predicted gene products showed sequence similarities, indicating that they may be orthologous (Supplementary Figs. 2d, f–h, and k–m). Likewise, adaptive radiation of ATLs within rice may be observed: groups a, d, and f harbor large clades with only rice proteins (see Fig. 2). We have also found tandem duplications in *ATL* genes in *Arabidopsis* and rice, suggesting that lineage-specific expansion may have led to the generation of some members of this family (Fig. 4 and data not shown). Two tandem genes that may have arisen from duplication events are *ATL10* and *ATL76*. For these two genes specialization seems to have occurred since a T-DNA insertion in *ATL10* renders a lethal phenotype (Table 1), while viable plants are obtained from a T-DNA line in ATL76. This suggests that there are functional differences between the two genes and supports the fact that new gene functions often evolve from gene duplication (Copley et al. 2003).

Another feature of the gene structure analysis is that about 90% of the *ATL* genes lack introns. This is confirmed by the analysis of cDNA clones for almost all of the *Arabidopsis ATL*s that are publicly available (see Supplementary Table 1). Noteworthily, most of the *ATL*s containing introns are clustered within group n, cDNA clones are also available for all of them, confirming the predicted gene structure (see Fig. 2 and Supplementary Table 1). Group n, which corresponds to a separate clade, is the only group that includes members from both *Arabidopsis* and rice. This fact suggests that these are genes of an ancient origin, which probably arose before the separation of mono- and dicotyledonous plants. A reverse-transcribed cDNA could be the origin of intronless genes (Maestre et al. 1995). If this is the case, the origin of intronless *ATL* genes may also have occurred before the separation between monocot and dicot plants since both species contain a high

number of putative orthologous intronless *ATL* genes. It is tempting to speculate that the general structure of the *ATL*s has then evolved as a basic functional module.

We have also carried out a survey of T-DNA insertions in 30% of the *Arabidopsis ATL* genes. Analysis of loss-of-function mutations is an important tool in the search for gene function. Loss-of-function mutations in *ATL* genes have not been previously described. Indeed, in comparisons of the wild-type and the different plants carrying an insertion in *ATL* genes, no obvious morphological variations were detected in any of the mutant lines growing under standard conditions. One of the reasons for the lack of detectable phenotypes is gene redundancy since structurally related proteins that clustered together were detected for almost every one of the corresponding mutated genes. Supporting this fact are the results for *ATL43* and *ATL4* insertional mutants. Neither of these genes seems to have a structurally related protein and their insertional mutations displayed a phenotype. The absence of evident phenotypes also suggests that *atl* mutants may exhibit subtle alterations that are traceable only under very specific conditions.

The ATL proteins are likely to be single-subunit E3 ubiquitin ligases since EL5, a member of the ATL family from rice, mediates in vitro autoubiquitination in a reaction depending on E1, E2, and ubiquitin. Additionally, nine of the RING-H2 proteins inferred in our survey to be ATLs possess in vitro ubiquitin ligase activity (Takai et al. 2002; Stone et al. 2005). Other components of ubiquitin ligases in *Arabidopsis* such as F-box, U-box, and RING proteins encode additional distinct domains; for instance, WD-40, LRR, or ARM domains are commonly encoded in F-box, U-box, and RING-finger proteins (Azevedo et al. 2001; Gagne et al. 2002; Mudgil et al. 2004; Stone et al. 2005). Apart from the distinct modules described for ATLs, no other well-conserved recognition or protein interaction domains were detected, suggesting that the structure of the ATL protein is sufficient to operate as a functional module and that specific function may arise from the regions of similarity that occur within putative carboxy-terminal domains of unknown function found in some of the ATLs (see Supplementary Fig. 2).

Four members of the family, *ATL4*, *ATL6*, *ATL8*, and *ATL10*, are likely to be essential for viability since homozygous plants for T-DNA insertion were not recovered. Analysis of expression revealed that *ATL8* is mainly expressed in young siliques, suggesting a role during embryogenesis. Mutant analysis on RING-H2 genes has been scarce. MsRH2-1, an alfalfa gene highly related to *ATL4*, when expressed ectopically in alfalfa or in *Arabidopsis*, renders dramatic and pleiotropic alterations in plant morphology and development; alterations in hormone response have been inferred for such a response (Karlowski and Hirsch 2003). RIE1, a non-ATL RING-H2-finger protein, shows an embryo-lethal phenotype. It encodes the RING-H2-finger domain at the carboxyl end, *RIE1* is expressed in all tissues tested, and embryo development is arrested from the globular to the torpedo stages (Xu and Li 2003). These observations indicate that ATL genes as well as other RING-H2 zinc-finger genes may play pivotal roles during early stages of plant development, linking these putative ubiquitin ligases to such processes.

Compelling evidence indicates that several ATLs may participate in defense response in plants. *ATL2* mRNA is rapidly and transiently induced after incubation with elicitors of pathogen response such as chitin and cellulases (Salinas-Mondragon et al. 1999). In *eca* mutants, which show constitutive expression of *ATL2* and other *ATL* genes, expression of defense-related genes is also impaired (see black squares in Fig. 2) (Serrano and Guzman 2004). *EL5*, a rice ATL (*OsATL24*), is also an elicitor-induced gene (Takai et al. 2001). Recently, it has been shown that induction of *ATL*s is not restricted to fungal-associated elicitors; significant induction of seven *ATL*s by bacterial flagellin, ranging between 1.9- and 30.2-fold, was reported (see gray squares in Fig. 2) (Navarro et al. 2004; Zipfel et al. 2004). Together these observations indicate that several *ATL*s may be part of the innate immune response mediated by pathogen molecules. This response, also known as PAMPs (pathogen-associated molecular patterns), is conserved among plants and animals (Nurnberger et al. 2004). A previously unidentified role for ATLs is also revealed in this work. A member of the ATL family may be in involved in the ABA response since the insertion line in *ATL43* shows an ABA-insensitive phenotype. This observation is supported by the fact that the expression of *ATL43* as well as other *ATL*s have been reported to be induced after ABA treatment in genomewide expression analysis (Hoth et al. 2002; Sanchez et al. 2004).

The function in plants for the enormous number of ubiquitin ligases within the RING-H2 class awaits unraveling. A two-hybrid screening detected interaction between a NAC (NAM/ATAF1/2/CUC2) transcription factor and the RING-H2 protein RHA2a (Greve et al. 2003). It was inferred that RHA2a regulates the activity of the NAC transcription factor since expression of β-glucuronidase fusions of both proteins colocalized to the nucleus. RHA2a is highly related to the ATL family but it lacks the highly conserved proline residue located at the central region of the RING-H2 domain (see P residue in Fig. 1). Indeed, RHA2a containing a mutation that adds the proline residue partially abolishes the

444

interaction with the NAC transcription factor, indicating that this residue is important for recognition (Greve et al. 2003). Further analysis of plant lines carrying a mutation in more than one *ATL*, analysis of the ectopic expression of *ATL* genes, and the search for possible targets of the putative ubiquitin ligases will help to unravel the role of this multigene family during the plant life cycle.

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