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# Isolation and Gene Expression Analysis of *Arabidopsis thaliana* Mutants With Constitutive Expression of *ATL2*, an Early Elicitor-Response RING-H2 Zinc-Finger Gene

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ABSTRACT

Genes with unstable transcripts often encode proteins that play important regulatory roles. *ATL2* is a member of a multigene family coding highly related RING-H2 zinc-finger proteins that may function as E3 ubiquitin ligases. *ATL2* mRNA accumulation occurs rapidly and transiently after incubation with elicitors of pathogen response. We screened 50,000 $M_2$ families from a line that carries a fusion of p*ATL2* to the GUS reporter gene and isolated five mutants, which we named *eca* (*e*xpresión *c*onstitutiva de *A*TL2), that showed constitutive expression of the reporter gene. One mutant exhibits a drastic stunted phenotype while the other four grow similarly to wild type. Two early chitin-induced genes and known pathogenesis-related genes such as *NPR1*, *PAL*, and *CHS* are activated in all the mutants whereas members of the ATL family and *PR-1* and *PDF2.1*, which are markers of the salicylic acid (SA) jasmonate (JA) defense-response pathways, display differential expression between the mutants. These observations indicate that the ECA gene products may function in the early steps of an elicitor-response pathway, although some of them may function at other stages on the SA or JA defense-response pathways. Likewise, the fact that *ATL2* and other members of the ATL family are activated in *eca* mutants links the induction of this putative class of ubiquitin ligases to plant defense signaling pathways.

PLANTS have acquired specialized means to perceive and act in response to the natural environment. Invading pathogens or abiotic stress trigger a response when detected by the plant. Basic constituents of microbial pathogens have been shown to elicit defense responses in plants; in such a way, the plant senses the presence of the invading pathogen (Nimchuk *et al.* 2003). These constituents, generally known as elicitors, are believed to be perceived by specific receptors (Shibuya *et al.* 1993; Ebel 1998; Day *et al.* 2001). Components of the cell wall of fungi are powerful and general elicitors in plants. An example of these are fragments of chitin (*N*-acetyl-chitooligosaccharides oligomers), for instance, released from the fungal cell wall by endochitinases (Stacey and Shibuya 1997). Various approaches have been followed to study the response to elicitors. Cell suspension cultures of rice (Yamada *et al.* 1993), soybean (Muller *et al.* 2000), tomato (Granado *et al.* 1995), and parsley (Hahlbrock *et al.* 1995) have provided a convenient system to analyze the induction of several cellular responses to chitin fragments. These responses include induction of a transient depolarization of membrane potential (Kuchitsu *et al.* 1993), the production of reactive oxygen species (Lee *et al.* 1999), increase in cytoplasmic calcium levels (Fellbrich *et al.* 2000), protein phosphorylation (Dietrich *et al.* 1990; Felix *et al.* 1991), and changes in gene expression (Minami *et al.* 1996; Nishizawa *et al.* 1999).

High-throughput analysis of gene expression has been used to analyze defense response in plants (Ramonell and Somerville 2002). Large-scale monitoring of gene expression has been reported in Arabidopsis and rice using expressed sequence tag (EST) microarrays. The Arabidopsis array used was composed of 2375 ESTs from which 71 responded to chitin treatment (Ramonell *et al.* 2002). The rice array consisted of 8987 ESTs from which 259 clones showed altered expression after treatment with *N*-acetylchytooctaose (Akimoto-Tomiyama *et al.* 2003). In both Arabidopsis and rice, genes presumably involved in signal transduction processes and in the response to pathogen attack were among the classes of genes that displayed altered response to the elicitor. Interestingly, several Arabidopsis genes showed a strong and very early induction after chitin treatment. This response was further analyzed in an attempt to unravel the mechanisms of response to this type of elicitor, concluding that the response to elicitor is a complex process that may consist of a network of signal transduction pathways (Zhang *et al.* 2002).

**This article is dedicated to the memory of the late Gilberto Mosqueda Cano.**

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We have previously shown that some RING-H2 zinc-finger genes are rapidly induced in response to elicitors (Salinas-Mondragon *et al.* 1999). These RING (*r*eally *i*nteresting *n*ew *g*ene)-finger genes are members of a gene family, named ATL, that shows various common structural features. In addition to a highly conserved RING-H2 zinc-finger domain they contain an amino-terminal transmembrane domain followed by a short basic region and a 16-bp conserved region (Salinas-Mondragon *et al.* 1999). EL5, a gene displaying similar features, has been described in rice; EL5 is also rapidly and transiently induced after elicitor treatment (Takai *et al.* 2001). The RING-finger motif is found ubiquitously in eukaryotes in a wide range of proteins (Freemont 1993; Borden and Freemont 1996). A common function has been inferred for several RING-finger domains. It has been frequently found as part of E3s, a component of the ubiquitin system responsible for selecting the substrate to be ubiquitinated and degraded (Jackson *et al.* 2000; Joazeiro and Weissman 2000). El5 has been shown to be able to mediate "*in vitro*" autoubiquitination. This catalysis is dependent on the RING finger and can be mediated by two ubiquitin-conjugating enzymes of the rice Ubc4/5 family (Takai *et al.* 2002). The three-dimensional structure of the RING-finger domain from El5 has been established by NMR spectroscopy; it is similar to the structure of other RING-finger domains previously determined (Katoh *et al.* 2003). In addition, NMR titration analysis identified the required residues necessary for the interaction of the RING finger with the E2 conjugating enzyme (Katoh *et al.* 2003). In tobacco cell culture, *ACRE-132*, a gene of the ATL family, is induced during defense responses; this gene was identified in a search for genes that are activated very early upon infection (Durrant *et al.* 2000).

*ATL2* is a RING-H2 gene that responds to elicitor. *ATL2* encodes an unstable transcript that responds to the elicitor within minutes; the transcript accumulation is transient, declining after 60 min of incubation (Martinez-Garcia *et al.* 1996; Salinas-Mondragon *et al.* 1999). *ATL2* (At3g16720) was also found in a cDNA microarray analysis directed to identify genes with unstable transcripts in Arabidopsis (Gutierrez *et al.* 2002). Arabidopsis plants transformed with a fusion of *pATL2/GUS* show a localized pattern of expression in the leaf primordia of young seedlings. This pattern changes after elicitor treatment, initially appearing at the shoot apical region and subsequently disseminating throughout the seedling (Salinas-Mondragon *et al.* 1999). Since early response genes are likely to have regulatory functions and are expected to play central roles in signal transduction pathways, we reasoned that mutants altered in *ATL2* expression may reveal important components of the early defense-response pathway in plants. In this work, we have isolated and characterized mutants that constitutively express *ATL2*. We found that these mutants may be affected at a similar stage in elicitor response but at a different stage in salicylic-acid (SA)- and jasmonic-acid (JA)-regulated responses, two key pathways that modulate the defense response.

## MATERIALS AND METHODS

**Growth conditions and mutant isolation:** C24 was the parental *Arabidopsis thaliana* ecotype utilized for mutant isolation. 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 kept at 4° for 4 days and then incubated at 24° during 7 days in a growth chamber under continuous light. Plants were grown to maturity in a Percival Scientific growth chamber (model AR-32L) at 22° with a 16/8-hr photoperiod.

A transgenic line harboring a fusion of the *ATL2* promoter to the β-glucuronidase reporter gene (*pATL2/GUS*) was previously reported (Salinas-Mondragon *et al.* 1999). Seeds were chemically mutagenized using ethyl methanesulfonate as previously described (Guzman and Ecker 1990). The progeny of 2883 $M_1$ lines were screened individually; 15–20 seeds from each line were germinated in MS media for 7 days under continuous light, and seedlings were pulled out from the media and placed into 24-well microtiter plates (Corning, Corning, NY); histochemical GUS assay was then performed. We screened for pools that presented seedlings exhibiting constitutive expression of GUS; 66 lines were initially selected. Ten plants from each individual line were propagated independently and tested again for the constitutive expression of GUS. Finally, we selected 5 homozygous plants that stably maintained the phenotype; each one was originated from an independent $M_1$ line.

**GUS expression assays:** Histochemical and fluorometric assays were performed as described (Jefferson 1987). Samples were placed in 50% glycerol and mounted on microscope slides; tissues were analyzed under a Leica MZ12 stereomicroscope. Protein concentrations were determined by the Bradford assay using a commercial kit (Bio-Rad Laboratories, Richmond, CA). The results presented are the mean of the measurements of three different samples. Cellulysin (Calbiochem, La Jolla, CA) and chitin (Sigma, St. Louis) treatments were performed as previously described (Salinas-Mondragon *et al.* 1999).

**Northern blot hybridization:** RNA was prepared from 7-day-old seedlings, which were incubated in a MS media or MS media containing 100 mg/ml cellulysin for 60 min as previously described (Salinas-Mondragon *et al.* 1999). Samples of 20 μg of total RNA were fractionated in a 1.4% agarose-formaldehyde gel. The probe utilized was an *ATL2* cDNA insert prepared by random priming (GIBCO BRL). Blot hybridization was done on nylon membranes (Hybond N+, Amersham, Buckinghamshire, UK) at 65° with a probe concentration of $10^6$ cpm $ml^{-1}$ as previously described (Ausubel *et al.* 1988). The autoradiogram was analyzed using the software Image J. 1.29x (http://rsb.info.nih.gov/ij/); the 28S ribosomal RNA was used as a loading control.

**Genetic analysis of *eca* mutants:** Each homozygous mutant was backcrossed twice to the parental line that carries the *pATL2/GUS* transgene. $F_1$ and $F_2$ progenies were analyzed for the segregation of the constitutive expression of the GUS; the phenotype was analyzed in 7-day-old seedlings. The segregation ratio was verified calculating the $\chi^2$ for an expected Mendelian segregation. Genetic complementation tests were performed in crosses between all five *eca* (*e*xpresión *c*onstitutiva de *A*TL2) mutants, and the constitutive expression of the reporter gene on the resulting $F_1$ seedlings was analyzed.

**Expression analysis by RT-PCR:** RT-PCR screen was used to determine whether there was altered expression of various studied genes in the different mutants compared to the parental line. Samples of total RNA from 7-day-old seedlings were isolated using Concert Plant RNA reagent (Invitrogen) and DNAse I treated. 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°, 2 min at 94°, 30–40 cycles of 30 sec at 94°, 45 sec at 54°, 1 min at 72°, and a final extension of 10 min at 72° (except for *ATL2* where 40 cycles were done). Amplification products were fractionated into a 1.0% agarose gel. For RT-PCR analysis of ATL family members, the primer sequences were *ATL2* (At3g16720), PM1 5′-GGACTAGTGG CGCGCCCGAATGCGCCGTTTGTTTA-3′ and PM2 5′-GAGCT CTCACCTACTCTCTTCTCCTCCC-3′; *ATL4* (At3g60220), PM3 5′-GCCATGGCGGACGAAAACTAC-3′ and PM4 5′-GCCATG GACCCCTGAGAG-3′; *ATL6* (At3g05200), PM5 5′-GAAGATCT TGAAGCTCCGATCATA-3′ and PM6 5′-GCTCTAGAAACCGG TAATCTCACCG-3′; *ATL8* (At1g76410), PM7 5′-GCCGTCTC TCGATGCGCTTGGC-3′ and PM8 5′-TCTTCACGTTGTTTGA TTCGGG-3′; *ATL11* (At1g72200), PM9 5′-CCCCAAAGGTAGA ACCAATCTC-3′ and PM10 5′-CACTCCCTAAGCTCCCGGT TCT-3′; *ATL12* (At2g20030), PM11 5′-GCTGTGTTTAAGAACC GCTGGA-3′ and PM12 5′-TTTGGCGTGTCGTGTTTAGGTC-3′; *ATL13* (At4g30400), PM13 5′-CCCTTGTTGGTGTAACTGT TGA-3′ and PM14 5′-GAGATGAAGGAGACCGGAGATG-3′; *ATL16* (At5g43420), PM19 5′-GTGGCTTCAGAACAACGCC AAT-3′ and PM20 5′-TCCAACCACCAGAACTTCGCGG-3′; ATL17 (At4g15975), PM21 5′-CATGCTCACCACCACAATC TTA-3′ and PM22 5′-CTTGCATGCTACTGACCGTCGG-3′. For RT-PCR analysis of pathogen-related genes, the primer sequences were *PDF1.2* (At5g44420), PM25 5′-ATGGCTAAG TTTGCTTCCAT-3′ and PM26 5′-TTAACATGGGACGTAAC AGA-3′; *NPR1* (At1g64280), PM27 5′-ATGGACACCACCATT GATGG-3′ and PM28 5′-TCACCGACGACGATGAGAGA-3′; *PR1* (At2g14580), PM29 5′-ATGTCTAGAACTGGCTATTCT CGATT-3′ and PM30 5′-TTTCTAGAGCTTCTCGTTCACAT AAT-3′; *PAL* (At2g37040), PM31 5′-ATGTCTAGATACGGGG CACACAAGAG-3′ and PM32 5′-TCTAGATATTGGAATGGG AGCTCCGT-3′; *CHS* (At5g13930), PM33 5′-ATGGGATCCGC TGGTGCTTCTTCTTT-3′ and PM34 5′-TTGGATCCGAACG CTGTGCAAGACGA-3′; For RT-PCR analysis of chitin-induced genes, the primer sequences were *AtMK3* (At3g45640), PM35 5′-ATGAACACCGGCGGTGGCCA-3′ and PM36 5′-CTAAC CGTATGTTGGATTGA-3′; zinc-finger (At1g27730), PM37 5′-ATGGCGCTCGAGGCTCTTAC-3′ and PM38 5′-GACCGGA AAGTCAAACCGAG-3′. For RT-PCR analysis, *CF150* (At1g72150) was used as constitutive control, and primer sequences were PM39 5′-CCGACAAGGAGAAGCTTAACAAGTT-3′ and PM40 5′-CGGCAGATTTGGATGGACCAGCAAG-3′. Primers used in RT-PCR assays were designed to span two exons, except for intronless genes. Quantitative analysis was made using the software Image J. 1.29x (http://rsb.info.nih.gov/ij/). Images were standardized using the *CF150* image as loading control. Data, shown as fold induction, represent the mean of the measurements of samples from two independent experiments; expression is relative to wild type. We did not determine the number of cycles where amplifications begin to plateau for each oligonucleotide pair but since we used 30 and 40 cycles we are most probably not in the linear range; the analysis is not semiquantitative.

## RESULTS

**Isolation of elicitor-response mutants:** Histochemical analysis of light-grown Arabidopsis seedlings transformed with a fusion of *pATL2/GUS* show a localized pattern of expression in the leaf primordia of young seedlings that is detected throughout the seedling after cellulysin or chitin treatment (Salinas-Mondragon *et al.* 1999). We exploited the response to these elicitors as a phenotype to screen for mutations that impair such a pattern. From 2883 $M_1$ lines we analyzed ~50,000 $M_2$ seeds (see materials and methods). The progeny of each plant were screened independently, searching for pools containing seedlings that exhibited constitutive expression of the GUS reporter gene in the absence of elicitor. We found 5 lines that reproducibly displayed such a phenotype. We named these mutant lines *eca1–eca5* (Figure 1). The five mutants represent independent mutations since they were identified from different plants. Before any analysis, the mutants were backcrossed twice to the *pATL2/GUS* parental line. We also screened for the reverse phenotype seedling that did not show induction of GUS expression after elicitor treatment, and we found one mutant. This mutant displayed a similar pattern of GUS expression with or without elicitor treatment (data not shown); analysis of this mutant is under way and is not considered here.

**Genetic analysis of *eca* mutants:** Segregation analysis was performed for the five *eca* mutants after crossing to the parental line. The $F_2$ progeny were analyzed for the constitutive expression phenotype. The patterns of Mendelian segregation indicated that in all cases the *Eca* phenotype was caused by a single recessive mutation (Table 1). To determine whether the mutants were affected at the same or at different loci, genetic complementation was analyzed; reciprocal crosses for all combinations were performed. The $F_1$ seedlings displayed a wild-type pattern of GUS expression, suggesting that they were not allelic mutations. The results indicated that each one of the mutations affects an independent locus (Table 2).

**Analysis of ATL2 expression in *eca* mutants:** Variations in the level of GUS activity were detected in the five mutants at the seedling stage. For instance, *eca2*, *eca3*, and *eca5* show less spreading of GUS and less GUS activity than the other two mutants (Figure 1). The increase in activity in the five mutants ranged from 2-fold (*eca3*) to 14-fold (*eca1*; Figure 1B, open bars). The response to elicitor treatment, either cellulysin or chitin, persisted in the mutants. Compared to the parental line that displayed a more than 3-fold increase after a 2-hr treatment, each of the *eca* mutants showed variation in the response to elicitor that ranges from 40 to 100% (Figure 1B, solid bars). An inverse correlation between the level of the constitutive expression of ATL2 and the response to elicitor seems to take place: the response to elicitor in the strongest constitutive mutants, *eca1* and *eca4*, was lower than that in the other mutants.

RNA gel blots were then employed to examine the expression of the endogenous *ATL2* in the *eca* mutants. There is at least a twofold increase in the level of *ATL2*

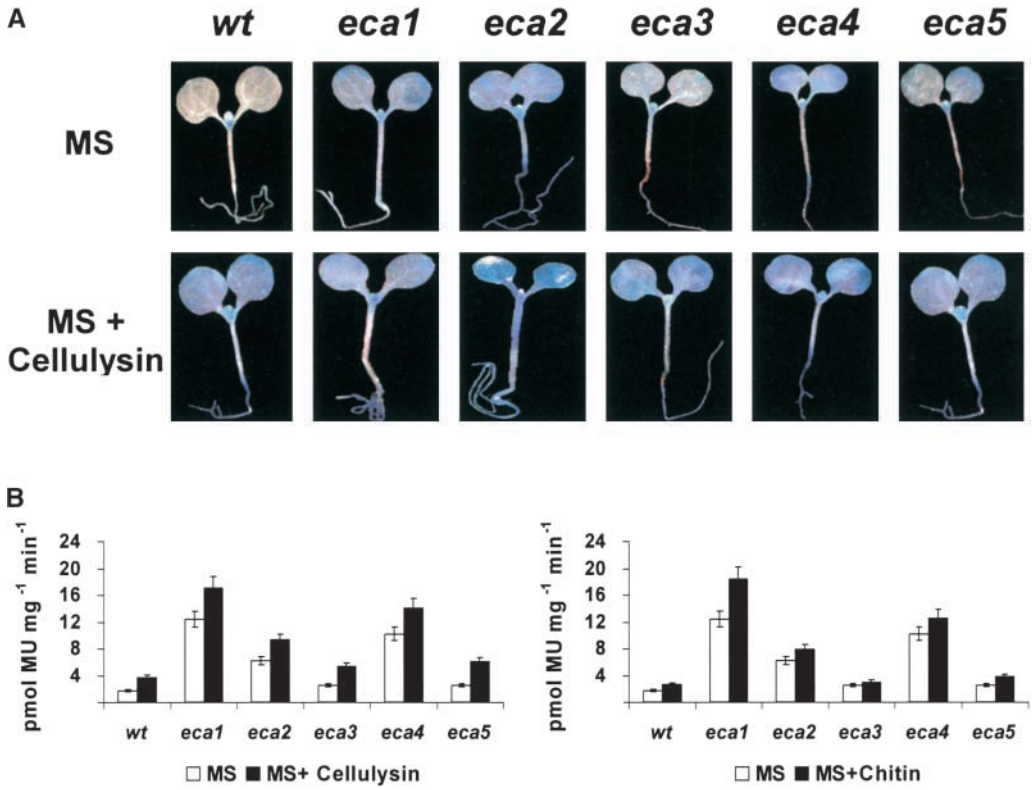


FIGURE 1.—Constitutive expression of *ATL2* phenotype in *eca* seedlings. (A) Histochemical localization of the GUS reporter gene. Seven-day-old light-grown seedlings germinated in MS media were incubated for 120 min in MS media or MS media containing 100 mg/ml cellulysin; representative samples are shown. (B) Fluorometric analysis of GUS activity from samples in A (left graph) and from samples incubated in a solution containing 100 mg/ml chitin (right graph); measurements are the mean of three independent samples.

transcript, with minor variations between the mutants (Figure 2, A and B, open bars). When seedlings were treated with elicitor, differences between the parental line and the mutants were more evident; these differences were between three- and eightfold. These observations confirm that the expression of *ATL2* is modified in the mutants and that differences in the constitutive response and in the response to elicitor exist between them (Figure 2, A and B). The level of endogenous *ATL2* mRNA is similar in all the mutants, whereas the activity of the GUS reporter driven by the *ATL2* promoter showed differences between the mutants. The *GUS* construct did not include the 3′ UTR of the *ATL2* transcript, which may include sequences that target mRNA for degradation (MARTINEZ-GARCIA *et al.* 1996). Thus, since *ATL2* encodes a highly unstable transcript, the inconsistency of the results may be explained by regulation of the level of mRNA accumulation by a post-transcriptional mechanism.

**Growth features in *eca* mutants:** We observed the effects of *eca* mutations on the growth of plants to maturity. Comparison of 7-day-old, light-grown parental and mutant lines exhibited no visible differences (Figure 3). In adult plants, only *eca1* displayed readily detectable

**TABLE 1**

**Genetic analysis of *eca* mutants**

| Cross (female × male) | Type | Total | Expression pattern | | $\chi^2$ |
|---|---|---|---|---|---|
| | | | wt | *eca* | |
| ECA1/ECA1 × eca1/eca1 | $F_1$ | 17 | 17 | 0 | |
| ECA1/ECA1 × eca1/eca1 | $F_2$ | 90 | 65 | 25 | 0.369; $P < 0.05$ |
| ECA2/ECA2 × eca2/eca2 | $F_1$ | 15 | 15 | 0 | |
| ECA2/ECA2 × eca2/eca2 | $F_2$ | 75 | 58 | 17 | 0.217; $P < 0.05$ |
| ECA3/ECA3 × eca3/eca3 | $F_1$ | 10 | 10 | 0 | |
| ECA3/ECA3 × eca3/eca3 | $F_2$ | 115 | 90 | 25 | 0.652; $P < 0.05$ |
| ECA4/ECA4 × eca4/eca4 | $F_1$ | 9 | 9 | 0 | |
| ECA4/ECA4 × eca4/eca4 | $F_2$ | 110 | 85 | 25 | 0.302; $P < 0.05$ |
| ECA5/ECA5 × eca5/eca5 | $F_1$ | 15 | 15 | 0 | |
| ECA5/ECA5 × eca5/eca5 | $F_2$ | 70 | 55 | 15 | 0.476; $P < 0.05$ |

$\chi^2$ was calculated for an expected 3:1, wild-type-to-mutant ratio.

**TABLE 2**

**Complementation analysis of *eca* mutations**

| | Recipient | | | |
|---|---|---|---|---|
| Donor | *eca2* | *eca3* | *eca4* | *eca5* |
| *eca1* | + | + | + | + |
| *eca2* | | +[a] | +[a] | +[a] |
| *eca3* | | | +[a] | +[a] |
| *eca4* | | | | +[a] |

+, complementation.
[a] The reciprocal cross was also tested.

morphological changes when compared to the other mutants or the parental line. Homozygous *eca1* plants were severely stunted when grown to maturity in soil and generated a small number of seeds (Figure 3). This stunted phenotype is either linked or very close to the *eca1* mutation, since after two backcrosses and after the analysis of ~100 progeny the constitutive expression of the GUS and the stunted phenotype segregated together (data not shown).

Expression analysis of the *pATL2/GUS* fusion in adult plants was determined to establish the effect of the *eca* mutation in ATL2 expression in mature organs. Expression was analyzed in 3-week-old rosette leaves, stems, and inflorescences (Figure 4). Differences in GUS staining and activity were observed when the five *eca* mutants and the wild-type line were compared (Figure 4B). Higher levels of GUS activity were detected in all organs of *eca1*, *eca2*, and *eca4* whereas activity similar to the parental line was observed in *eca3* and *eca5*. This effect was readily perceived in inflorescences; weak histochemical GUS was observed in the parental, *eca3*, and *eca5* lines compared to the other mutants. These results were consistent with the GUS activity measured in seedlings where the *eca3* and *eca5* showed the least increase of GUS activity (Figure 1). Likewise, *eca4* displayed a strong expression at both seedling and adult stages. The differences of ATL2 expression in the *eca* mutants suggest that they are altered at different stages of the response.

**The expression of early chitin-induced genes is activated in all the *eca* mutants:** DNA microarray experiments revealed several genes in Arabidopsis that responded rapidly and strongly to chitin (Ramonell *et al.* 2002; Zhang *et al.* 2002). Since *ATL2* responded to chitin, we wanted to test whether the expression of other genes that responded to chitin was also affected in the *eca* mutants. The expression of two of these genes was tested: a zinc-finger protein (At1g27730) and a mitogen-activated protein (MAP) kinase (At3g45640; Zhang *et al.* 2002). These genes were selected because mRNA accumulation occurs within 30 min of chitin treatment

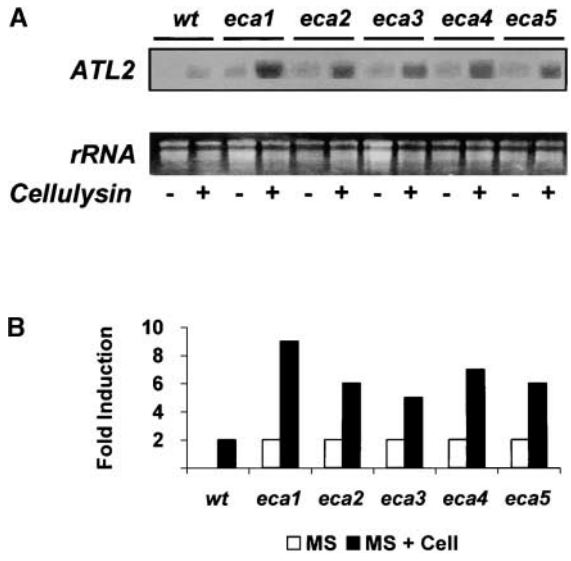


Figure 2.—mRNA accumulation of *ATL2* increases in *eca* mutants. Total RNA was isolated from 7-day-old light-grown seedlings that were incubated for 60 min in MS media or MS media containing 100 mg/ml cellulysin. (A) A total of 20 μg of total RNA were fractionated in 1.4% formaldehyde gel, blotted onto nylon membranes, and hybridized under high stringency with $^{32}$P-labeled *ATL2* probe. (B) Quantification of data shown in A relative to the 26S ribosomal RNA. Data presented as fold-induction are the relative values between mutant and wild-type expression.

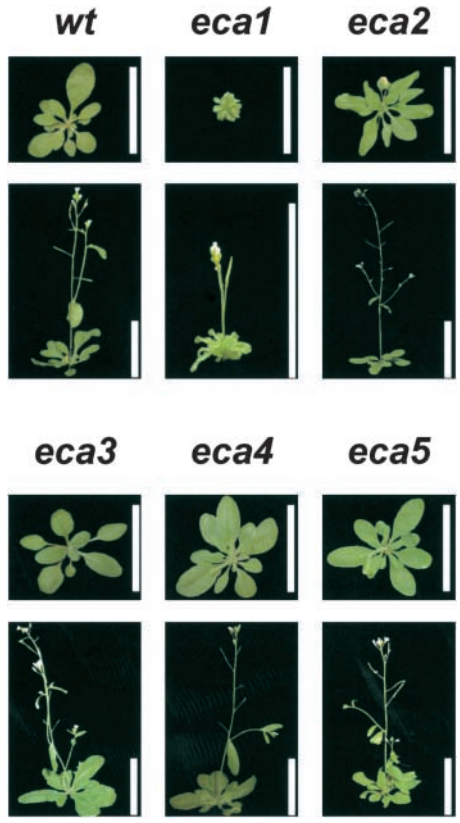


Figure 3.—Morphological changes in *eca* mutants. Plants were grown in a growth chamber (Percival Scientific) under a 16/8-hr photoperiod at 22°. Representative samples are shown. Bar, 5 cm.

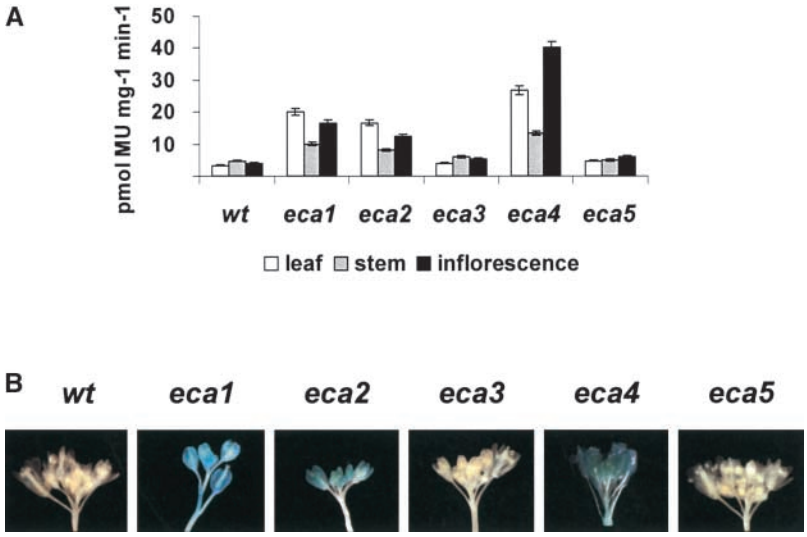


FIGURE 4.—Constitutive expression of *ATL2* phenotype in adult *eca* tissues. (A) Fluorometric analysis of GUS activity from tissues of 4-week-old plants. Results are the mean of the measurements of three different samples. (B) Histochemical localization of GUS of samples shown in A; representative inflorescences are shown.

and each of them showed a different response when its dependence on various defense pathways was examined. Our analysis (with no chitin added for induction) indicated that transcript levels from both genes showed elevated steady-state levels in the *eca* mutants compared to the wild-type plant, with little or no difference between the *eca* mutants (Figure 5). A more notable difference was observed for the zinc-finger protein than for the MAP kinase. This difference correlates with the basal level of expression reported for both genes and with the relative level of induction in response to chitin: the zinc-finger gene is strongly induced by chitin and showed less basal expression than the MAP kinase gene (ZHANG *et al.* 2002). The fact that two chitin-regulated genes are activated in the five *eca* mutants indicates that all the mutants are affected in a chitin-responsive signaling pathway.

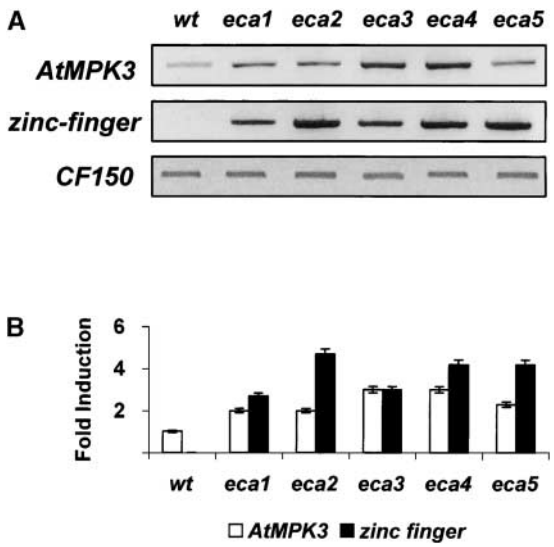


FIGURE 5.—Induction of early chitin-response genes in *eca* mutants. (A) RT-PCR assays were performed from total RNA isolated from 7-day-old light-grown seedlings and treated with DNAse I; 100 ng of RNA was used in each reaction. Samples were fractionated in a 1.0% agarose gel; the size of PCR products are 1113 bp for *AtMK3* (At3g45640) and 669 bp for a zinc-finger protein (At1g27730). (B) Quantification of data shown in A relative to *CF150* (At1g72150) as a control of constitutive expression. Data presented are the mean of two independent experiments. Fold-induction represents the relative value between the expression of the mutant and the parental line.

**The *eca* mutants display alterations in expression of members of the ATL family:** To determine the specificity of the constitutive expression of *ATL2* in the *eca* mutants, we tested the expression of 15 members of the ATL family. Five genes, including *ATL2*, corresponded to previously described *ATL* genes (*ATL3*, *ATL4*, *ATL5*, and *ATL6*; SALINAS-MONDRAGON *et al.* 1999); the additional 10 were obtained from databases. These genes were selected as members of the ATL family having conserved RING-H2 zinc finger in the central region of the protein as well as three other expected domains toward the amino-terminal end: a hydrophobic, a basic, and a conserved sequence (Figure 6A). By using RT-PCR, we detected differential expression of 9 of these genes (Figure 6B). Seven of them (*ATL2*, *ATL6*, *ATL8*, *ATL12*, *ATL13*, *ATL16*, and *ATL17*) were expressed in all the mutants, compared to reduced or no expression in the parental line. It is noteworthy that in four of them (*ATL6*, *ATL12*, *ATL13*, and *ATL16*) a higher expression was detected in *eca1* and *eca4* mutants (compare the top two graphs in Figure 6C), which are the mutants exhibiting a strong activation of the *pATL2/GUS* (see Figures 1 and 2). The other two genes (*ATL4* and *ATL11*) were expressed only in the *eca1* mutant (Figure 6C, bottom graph), which is the one having a dramatic stunted phenotype (see Figure 3).

**Analysis of defense-response gene expression in *eca* mutants:** To determine whether the constitutive expression of *ATL2* in the *eca* mutants was linked to the plant response to pathogens, the level of various previously described defense-response genes was assessed. We se-

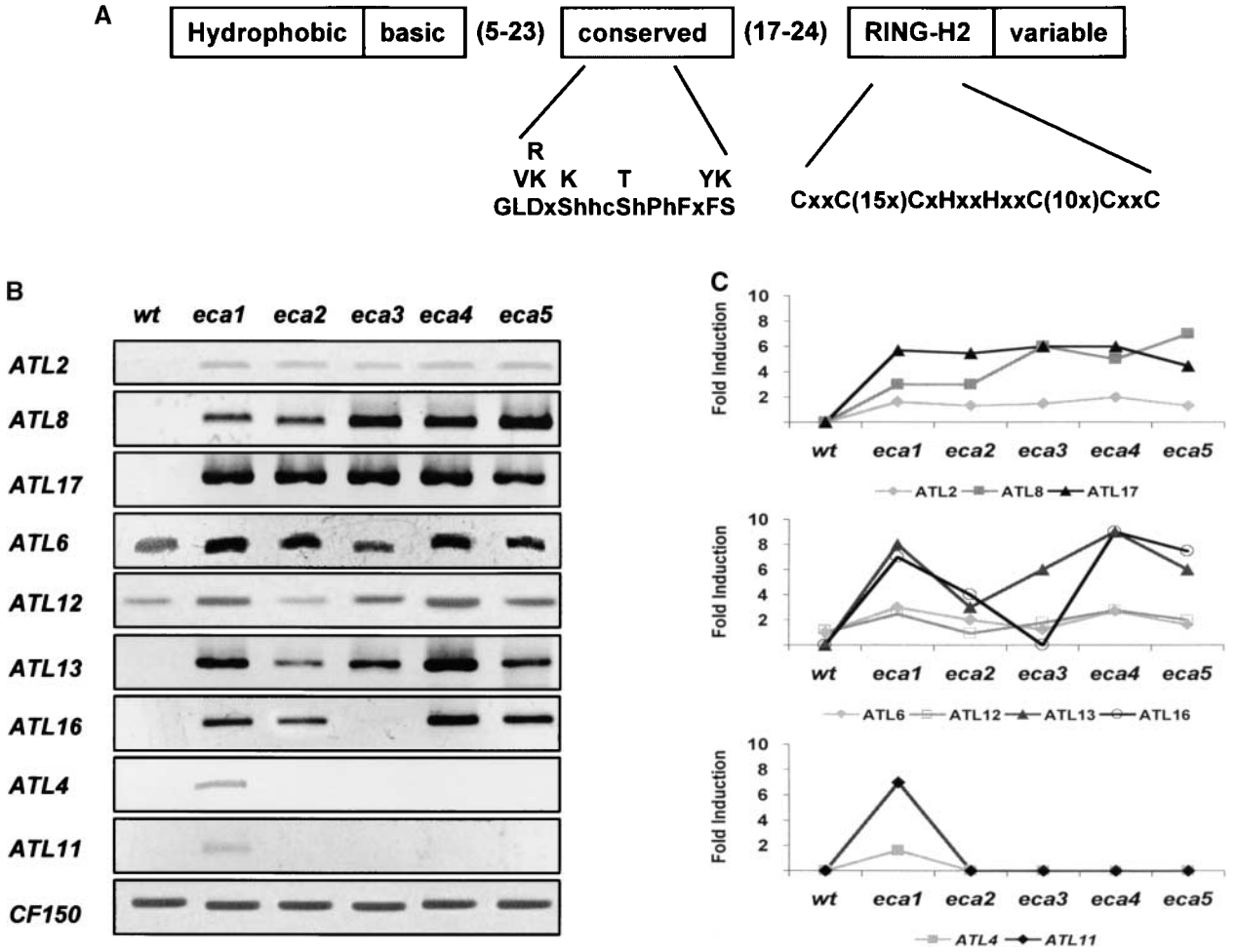


FIGURE 6.—Induction of the expression of members of the ATL family in *eca* mutants. (A) Representation of common features present in ATL proteins; the diagram is based on the amino acid sequences of the nine ATLs shown in B: *ATL2* (At3g16720), *ATL4* (At3g60220), *ATL6* (At3g05200), *ATL8* (At1g76410), *ATL11* (At1g72200), *ATL12* (At2g20030), *ATL13* (At4g30400), *ATL16* (At5g43420), and *ATL17* (At4g15975). (B) RT-PCR assays were performed from total RNA isolated from 7-day-old light-grown seedlings and treated with DNAse I; 100 ng of RNA was used in each reaction. The sizes of the RT-PCR products were *ATL2*, 500 bp; *ATL4*, 981 bp; *ATL6*, 1197 bp; *ATL8*, 398 bp; *ATL11*, 935 bp; *ATL12*, 394 bp; *ATL13*, 441 bp; *ATL16*, 496 bp; and *ATL17*, 706 bp. Samples were fractionated into a 1.0% agarose gel. Amplification of the following *ATL* genes was not detected: *ATL3* (At1g72310), *ATL5* (At3g62690), *ATL9* (At2g35000), and *ATL10* (At1g49220; data not shown). Similar levels of expression were detected in the mutant and the parental line of the following ATLs: *ATL14* (At4g30370) and *ATL15* (At1g22500; data not shown). (C) Quantification of data shown in B relative to *CF150* (At1g72150) as a control of constitutive expression. Data presented are the mean of two independent experiments. Fold-induction represents the relative value between the expression of the mutant and the parental line.

lected several known defense-response genes from which *PR-1*, *NPR1*, *PDF1.2*, *PAL*, and *CHS* showed differences in expression between mutant and parental lines. For *PR-1* and *NPR1*, implicated in the SA-dependent defense pathway (KLESSIG *et al.* 2000; SHAH 2003), no expression was detected in the parental line but differences in expression were observed in the mutants. Increase in *PR-1* was detected only in *eca1*, whereas *NPR1* was detected in all the mutants. *NPR1* functions upstream of *PR-1*; thus *eca* mutants may affect the activity of *NPR1* on *PR-1* in a different manner (Figure 7).

*PDF1.2* was selected as a marker for the JA response (TURNER *et al.* 2002). *PDF1.2* increased to a greater extent in *eca2* and *eca4* and was maintained at a similar level in the parental line and in the other mutants (Figure 7). This observation suggests that these two *ECA* genes may have a role in the JA-dependent defense pathway. They may negatively regulate a JA-dependent process, or alternatively, they may regulate a different physiological process that, when altered, results in upregulation of JA responses. *PAL* and *CHS* were tested for stress and late plant-defense-associated responses (DONG *et al.* 1991). Increased expression of these two genes was observed in the five *eca* mutants, indicating that the *ECA* genes may have a role in defense response.

Since the expression of various defense-response

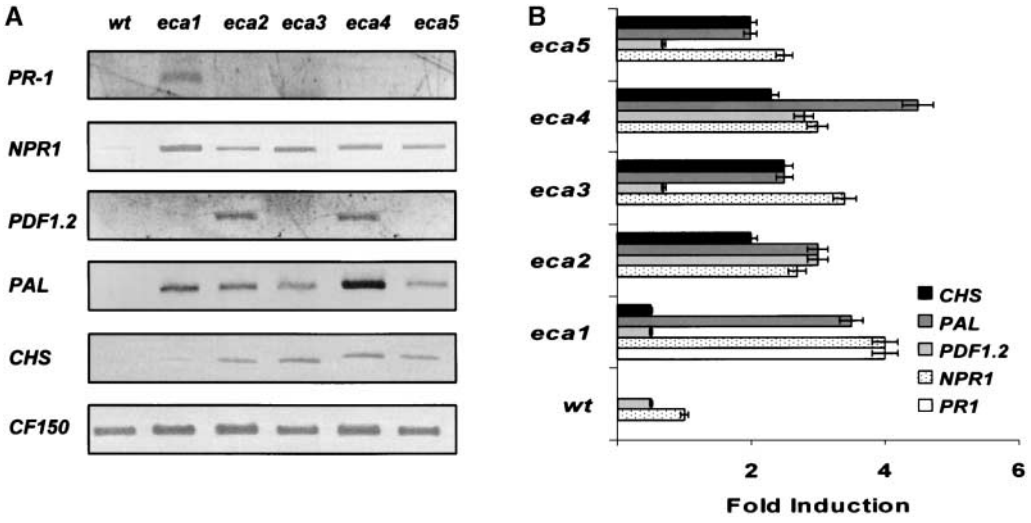


FIGURE 7.—Expression pattern of pathogenesis-related genes are modified in an *eca* background. RT-PCR assays were performed from total RNA isolated from 7-day-old light-grown seedlings and treated with DNAse I; 100 ng of RNA was used in each reaction. The size of the RT-PCR products are *PR1* (At2g14580), 490 bp; *NPR1* (At1g64280), 1782 bp; *PDF-1.2* (At5g44420), 243 bp; *PAL* (At2g37040), 2178 bp; and *CHS* (At5g13930), 1188 bp. Samples were fractionated in a 1.0% agarose gel. (B) Quantification of data shown in A relative to *CF150* (At1g72150) as a control of constitutive expression. Data presented are the mean of two independent experiments. Fold-induction represents the relative value between the expression of the mutants and the parental line.

genes was affected in the *eca* mutants, we looked in our mutants for possible physiological alterations often related to the defense response in plants. Under our growth conditions, we did not detect any type of spontaneous lesions in the leaves of the mutants (data not shown). This type of lesion is common in mutants that constitutively express a hypersensitive response related to localized cell death (DIETRICH *et al.* 1994). Attempts to detect callose accumulation by anilin blue staining were unsuccessful (data not shown); callose is deposited in cell walls as a response to pathogen attack (NISHIMURA *et al.* 2003).

## DISCUSSION

With the purpose of expanding the knowledge of the early responses of plants against environmental stresses, we set out to isolate mutants of Arabidopsis showing alterations in the expression of *ATL2*, an early elicitor-response RING-H2 zinc-finger gene. Related approaches, based on the constitutive expression of reporter genes from strong systemic acquired resistance (SAR)-inducible promoters in chimeric lines, have been previously directed to the isolation of mutants involved in disease resistance in plants (BOWLING *et al.* 1994; MALECK *et al.* 2002). Our approach uses a gene that encodes an unstable transcript that responds very rapidly and transiently to elicitors of the pathogen response. We reasoned that the mutants isolated by this means, which we named *eca*, might reveal novel components participating at the onset of the defense response in plants. In fact, most of the *eca* mutants are not developmentally altered. This is in contrast to the defense-related mutants previously identified, suggesting that these particular *eca* mutants may not affect a broad range of defense responses. Our results indicate that the *eca* mutants may define negative regulators of the defense response since all five mutations were recessive. Complementation analysis indicates that five independent loci were identified, indicating that it was not a saturating screen and that ATL2 may be under a complex signaling network. Modified expression of *ATL2* and other *ATL* genes as well as of known defense-response genes also takes place in these *eca* mutants, linking the induction of this putative class of ubiquitin ligases to plant defense signaling pathways.

The five *eca* mutants displayed an increase in the level of expression of the endogenous *ATL2*, demonstrating that the *pATL/GUS* fusion reproduced the expression of the endogenous *ATL2*. The increase in expression is not saturated in any of the mutants, since *ATL2* continues to respond to the elicitor, suggesting that a complete activation of *ATL2* may have a deleterious effect on plant growth or that several factors may contribute to its regulation. Unique and complex pathways for chitin perception and signal transduction have been inferred (ZHANG *et al.* 2002). Since *ATL2* was described as an elicitor-induced gene (SALINAS-MONDRAGON *et al.* 1999), the constitutive expression in all *eca* mutants of two known early chitin-induced genes (a zinc-finger protein and a MAP kinase; see Figure 5) indicates that a common feature of *eca* mutants is an alteration in a chitin response pathway.

On the basis of gene expression analysis and developmental alterations, it is possible to infer classes of *eca* mutants. For instance, *eca1* exhibited unique characteristics: in addition to a readily detectable stunted phenotype, it is the only mutant that shows an increase in the expression of the *PR-1* gene and of two *ATL* genes. The stunted phenotype was not unexpected since morphological changes are often associated with mutants affected by defense responses. A number of mutants, like those in the Arabidopsis *cpr* and *cim* class with a constitutive salicylic-acid-dependent signaling pathway, also dis-

play a stunted phenotype; these mutants were isolated by a similar strategy, selecting for the constitutive expression of a reporter gene driven by a promoter of a pathogenesis-related gene (Bowling *et al.* 1994; Maleck *et al.* 2002). These mutants show elevated levels of *PR-1* and many of them display lesion-mimic phenotypes portraying the hypersensitive response. Thus, there is a resemblance between the *eca1* mutant and the *cpr* and *cim* class of mutants: they have a stunted phenotype and increased expression of the *PR-1* gene. These observations indicate that ECA1 function might also act upstream of PR1. *eca2*, *eca3*, *eca4*, and *eca5* represent a different class of mutants also affected in the expression of defense-response genes; they did not show an increase in *PR-1* expression, but other defense-related genes were activated (see Figure 7). Many of the *cim*, *cpr* class of mutants are stunted and lesion mimic. The fact that *eca2*, *eca3*, *eca4*, and *eca5* are neither developmentally altered nor lesion mimic suggests that they are not broad-spectrum mutants and that they may be affected at specific steps of the defense response.

NPR1 is a key regulator of the systemic acquired resistance response, functioning upstream of *PR-1* (Klessig *et al.* 2000; Shah 2003). The fact that *NPR1* was activated in all the *eca* mutants indicates that ECA gene products may operate upstream of NPR1. Considering the different effect of *eca1* and the other *eca* mutants on *PR-1* expression, it is likely that these groups of mutants affect NPR1 activity in a different way. NPR1 functions in the nucleus by recruiting TGA transcription regulators to promoters of SAR-related genes; it is possible that NPR1 is active in *eca1* to induce *PR-1* transcription but is not active in the other *eca* mutants. Recently, it has been established that in the absence of SAR, NPR1 is located in the cytoplasm as an oligomer. Upon SAR induction, NPR1 is converted to a monomer that is able to get into the nucleus and regulate PR expression (Mou *et al.* 2003).

*PDF1.2* was activated the most in *eca2* and *eca4*. *PDF1.2* encodes a plant defensin with antimicrobial properties that is induced as a result of the jasmonate-dependent defense pathway; JA is one of a number of secondary signal molecules that amplify the defense responses (Turner *et al.* 2002). The JA and the SA signaling pathways can function in an antagonistic manner; there is evidence indicating that SA inhibits JA signaling and vice versa. On the other hand, some genes can be induced by either SA or JA, or both (Reymond and Farmer 1998; Glazebrook *et al.* 2003). Our analysis suggests that ECA2/ECA4 and ECA1 function in different pathways—ECA2 and ECA4 in the JA pathway and ECA1 in the SA pathway—although they may function in a common pathway that is independent of NPR1, as suggested for SSI1 (Shah *et al.* 1999). The effect may also be foreseen as indirect: the ECA proteins may regulate totally different processes that, when affected, result in upregulation of JA or SA responses. Two genes of secondary metabolism showed expression in all *eca* mutants. *PAL* and *CHS* are stress response genes that are induced upon pathogen attack (Glazebrook *et al.* 2003); the constitutive expression of these two genes supports the fact that *eca* mutants have alterations in defense-response mechanisms.

Many RING-H2 proteins function as part of the E3 ubiquitin ligases (Jackson *et al.* 2000; Joazeiro and Weissman 2000). EL5 is an elicitor-induced gene from rice that is a member of the ATL family (Takai *et al.* 2001). Although the molecular function of ATL genes is not known, the fact that the rice El5 protein mediates *in vitro* autoubiquitination suggests that other ATLs may function in ubiquitination as E3s as well (Takai *et al.* 2001; Katoh *et al.* 2003). Protein degradation mediated by ubiquitination plays key regulatory roles during several plant growth and developmental events and has been implicated in plant defense responses (Hare *et al.* 2003). The yeast SGT1 interacts with a component of the Skp1-Cullin-F-box ubiquitin ligase, mediating degradation of cell-cycle proteins (Kitagawa *et al.* 1999). The function of the SGT1 gene is conserved in plants, having a role in the resistance-gene-mediated defense response (Austin *et al.* 2002; Azevedo *et al.* 2002). It is reasonable to presume a role for ATL proteins in the control of protein abundance during plant defense response. ATLs may mediate ubiquitination of negative regulators of the defense response, thereby activating a certain aspect of the response. ATLs contain a highly predictable membrane-spanning domain and thus may target membrane proteins for degradation (Salinas-Mondragon *et al.* 1999). Differential expression of *ATL* genes was found in *eca* mutants. For instance, *ATL4* and *ATL11* were detected only in *eca1*, the only mutant that shows *PR-1* activation. These two *ATL* genes may target in proteins involved in the SA-dependent signaling pathway; possible targets may be proteins that modulate the expression of *CPR* or *CIM* genes that promote disease resistance (Clarke *et al.* 2001; Maleck *et al.* 2002).

Complex and diverse cascades of signaling networks govern the defense response in plants (Staskawicz *et al.* 2001; Kunkel and Brooks 2002; Ramonell and Somerville 2002). Specific recognition between the plant and the pathogen and broad mechanisms of pathogen recognition are both part of the defense response. The analysis of genes that respond early in the signaling cascade is a helpful resource to unravel the onset of the response. The analysis of the *eca* mutants reveals that a group of *ATL* genes may have a role in the expression of defense-response genes. Although the function of *ATL* genes is totally unknown, differential activation observed in the mutants provides a valuable tool to analyze the response at the transcriptional level. The confrontation of the mutants with several fungal and bacterial pathogens and the analysis of the genetic relationship with known mutants affected in disease resistance will confirm the participation of *eca* mutants in the

defense response and will place them more accurately in a signaling cascade.

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