The LeATL6-Associated Ubiquitin/Proteasome System May Contribute to Fungal Elicitor-Activated Defense Response via the Jasmonic Acid-Dependent Signaling Pathway in Tomato

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The expression of *LeATL6*, an ortholog of *Arabidopsis* *ATL6* that encodes a RING-H2 finger protein, was induced in tomato roots treated with a cell wall protein fraction (CWP) elicitor of the biocontrol agent *Pythium oligandrum*. The *LeATL6* protein was expressed as a fusion protein with a maltose-binding protein (MBP) in *Escherichia coli*, and it catalyzed the transfer of ubiquitin to the MBP moiety on incubation with ubiquitin, the ubiquitin-activating enzyme E1, and the ubiquitin-conjugating enzyme E2; this indicated that *LeATL6* represents ubiquitin ligase E3. *LeATL6* expression also was induced by elicitor treatment of *jai1-1* mutant tomato cells in which the jasmonic acid (JA)-mediated signaling pathway was impaired; however, JA-dependent expression of the basic *PR-6* and *TPI-1* genes that encode proteinase inhibitor II and I, respectively, was not induced in elicitor-treated *jai1-1* mutants. Furthermore, transient overexpression of *LeATL6* under the control of the *Cauliflower mosaic virus* 35S promoter induced the basic *PR6* and *TPI-1* expression in wild tomato but not in the *jai1-1* mutant. In contrast, *LeATL6* overexpression did not activate salicylic acid-responsive acidic *PR-1* and *PR-2* promoters in wild tomato. These results indicated that elicitor-responsive *LeATL6* probably regulates JA-dependent basic *PR6* and *TPI-1* gene expression in tomato. The *LeATL6*-associated ubiquitin/proteasome system may contribute to elicitor-activated defense responses via a JA-dependent signaling pathway in plants.

Additional keywords: *ATL* gene family, induced resistance, Micro-Tom.

Protein degradation in various biological processes such as transcription, signal transduction, and peroxisome biogenesis often is mediated by the ubiquitination pathway (Saurin et al. 1996). In addition, ubiquitin-mediated proteolysis also has been implicated in plant-signaling pathways for responses to phytohormones, photomorphogenesis, sucrose, and pathogens (Devoto et al. 2003; Ellis et al. 2002). Proteolysis of the target protein by the ubiquitination pathway involves three steps: the activation of ubiquitin via the formation of a thioester bond between the target protein and a cysteine at the active site of a

ubiquitin-activating enzyme (E1), the transfer of the activated ubiquitin to a ubiquitin-conjugated enzyme (E2), and the recognition of the target protein as a substrate by a ubiquitin-protein ligase (E3) (Hershko and Ciechanover 1998; Smalle and Vierstra 2004). The ubiquitinated protein is rapidly degraded via the 26S proteasome. In the sequential reaction of the three enzymes in the ubiquitination pathway, E3 plays a central role in the recognition of the target protein for ubiquitination.

In plants, E3 ubiquitin ligases are classified into various groups based on the presence of really interesting new genes (RING), HECT, or U-box domains (Vierstra 2003). The RING zinc-finger domain is described as a new class of zinc-finger proteins and is a major part of the E3 ubiquitin ligases (Borden and Freemont 1996; Saurin et al. 1996). The RING zinc-finger domain proteins function in hormone, light, and developmental processes; several target proteins for these processes, including COP1, SINAT5, and RMA1, have been identified (Vierstra 2003). SKP CDC53p/CUL1 F-box (SCF) complexes belong to a family of RING-type E3 ubiquitin ligases (Weissman 2001). The suppressor of G2 allele of SKP1 (*SGT1*), which was identified as essential defense signaling component, interacts with the required for Mla resistance 1 (*RAR1*) and regulates the activity of the SCF complex. *SGT1* and *RAR1* are required for the resistance (*R*)-gene-mediated resistance to pathogens in plants; therefore, SCF-mediated ubiquitination seems to be an important regulator of the *R*-gene-mediated resistance (Austin et al. 2002; Azevedo et al. 2002; Liu et al. 2002; Muskett et al. 2002; Peart et al. 2002; Shirasu et al. 1999; Tor et al. 2002; Toreno et al. 2002). Subsequent studies using various plant–pathogen combinations provide accumulating evidence indicating the important role played by the ubiquitin/proteasome system in the defense responses against pathogens. Recently, it has been demonstrated that the RIN2/RIN3 RING E3 ligases, which interact with the *RPM1*-encoding coiled coil-nucleotide binding site-leucine-rich repeat (LRR)-type R protein, act on a substrate that regulates *RPM1*- and *RPS2*-dependent hypersensitive response (HR) to *Pseudomonas syringae* carrying *avrRpm1* or *avrRpt2* (Kawasaki et al. 2005). In the *Cf-9*- and *Cf-4*-dependent HR in tomato and tobacco, it has been demonstrated that *Avr9/Cf-9* rapidly elicited (*ACRE*) genes that encode U-box E3 ubiquitin ligases are essential for defense responses (Durrant et al. 2000; Gonzalez-Lamothe et al. 2006; Yang et al. 2006).

ATL also belongs to a RING zinc-finger gene family in *Arabidopsis thaliana*, and it contains an amino-terminal transmem-

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brane domain followed by a short basic region and a 16-amino-acid residues-conserved region, except for a highly conserved RING-H2 zinc-finger domain (Salinas-Mondragon et al. 1999). Among the members of the *ATL* gene family, the *ATL2* and *ATL6* genes are rapidly induced after treatment with a chitin elicitor (Salinas-Mondragon et al. 1999). In addition, it has been demonstrated that, in rice, the expression of an ortholog of the *ATL* gene family, namely, the elicitor-responsive 5 (*EL5*) gene, also is upregulated at the early stage of elicitor treatment (Durrant et al. 2000; Takai et al. 2002). However, the function of elicitor-responsive *ATL* in the activation of defense responses has not been well established thus far.

Essential pathogen components such as cell wall fragments, enzymes, and secreted peptides exhibit defense response-inducible general elicitor activity; these components have been recognized as invariant pathogen-derived determinants called pathogen-associated molecular patterns (PAMPs) (Hammond-Kosack and Parker 2003; Nürnberger et al. 2004). Elicitins are well-studied sterol-binding proteins and are common PAMPs produced by many *Phytophthora* and *Pythium* spp. (Kamoun et al. 1997; Panabieres et al. 1997; Tyler 2002). Multiple sequence alignments and phylogenetic analyses of elicitors and elicitor-like proteins suggest the presence of at least five distinct classes within the elicitor family (Kamoun et al. 1997). Oligandrin is an elicitor-like protein with a molecular mass of approximately 10 kDa and is secreted by the nonpathogenic organism *Pythium oligandrum* (Picard et al. 2000). A conclusive study of oligandrin-induced resistance against *Phytophthora parasitica* in tomato has suggested that oligandrin triggers cytological and biochemical modifications in tomato cells, thereby inducing resistance to *P. parasitica* (Picard et al. 2000). In addition to oligandrin, Takenaka and Kawasaki (1994) have reported glycoprotein elicitor in the cell wall protein fraction (CWP) of *Pythium oligandrum*. CWP possesses two major proteins, POD-1 and POD-2, that have molecular masses of approximately 28 and 24 kDa, respectively. The amino acid sequences deduced from the corresponding cDNA sequences of *POD-1* and *POD-2* (DDBJ accession numbers AB217820 and AB217821) suggest that these proteins contain O-linked glycosylation sites and elicitor signatures that are the conserved motif of elicitor and elicitor-like proteins of *Phytophthora* and *Pythium* spp. listed in the BLOCK motif database (Picard et al. 2000; Ponchet et al. 1999; Takenaka et al. 2006). The purified CWP and mycelial homogenate of *P. oligandrum* that contained CWP induced defense reactions against pathogens in sugar beet and wheat (Takenaka et al. 2003). Furthermore, purified POD-1 and POD-2 each were sufficient to induce resistance to sugar beet seedling disease caused by *Aphanomyces cochlioides* and expression of several defense-related genes, indicating that POD-1 and POD-2, and also CWP containing them, appeared to have elicitor activity (Takenaka et al. 2006). However, the molecular mechanism underlying recognition of plants to CWP and the downstream signaling pathway for induction of the defense-related gene expression remain to be elucidated.

A global analysis of gene expression in tomato roots treated with CWP by using 11,520 nonredundant cDNA arrays indicated that the expression of approximately 250 genes was altered in the CWP-treated tomato roots (Takahashi et al. in press). Of upregulated 144 genes, defense-related genes (e.g., the basic pathogenesis-related [PR] protein [*PR-6*] that encodes proteinase inhibitor II and basic *PR-2* that encodes β -1,3-glucanase, which generally are regulated by jasmonic acid [JA]- and ethylene-mediated signaling pathways) were included. Other JA- and ethylene-related genes also were included in the upregulated genes in the CWP-treated tomato roots. The expression of genes that encode an ethylene receptor homolog; an ethylene-responsive transcription factor; an alleneoxide synthase, and a

lipoxigenase for JA biosynthesis; and three JA-inducible transcription factors (i.e., JERF1, NAC transcription factor JA2, and MYC transcription factor JA3) clearly were induced in CWP-treated tomato roots. This indicated that JA- and ethylene-dependent signaling pathways appeared to be activated in the CWP-treated tomato roots (Takahashi et al. 2006). However, the expression of acidic *PR* genes, which generally is regulated via a salicylic acid (SA)-dependent signaling pathway, was not observed in the CWP-treated tomato roots.

While analyzing the putative function of these upregulated gene products in CWP-treated roots, we observed that the expression of one gene encoding the RING-H2 protein was highly induced in tomato roots due to CWP treatment. This gene has been characterized in the present study. Comparative analysis of the amino acid sequences of the RING-H2 protein with other RING-finger proteins and the measurement of the ubiquitin ligase activity of the RING-H2 protein indicated that this protein may be an ortholog of *ATL6*, a ubiquitin ligase (E3) of *Arabidopsis thaliana*. Furthermore, the transient overexpression of this RING-H2 gene (*LeATL6*) in leaves of wild tomato and the *jai1-1* mutant tomato suggested that the upregulation of *LeATL6* may induce JA-dependent defense-related gene expression in CWP-treated tomato.

RESULTS

Upregulation of *LeATL6* in tomato roots treated with CWP or mycelial homogenate of *P. oligandrum*.

When tomato roots were treated with CWP, accumulation of the *LeATL6* transcript was observed 4 h after treatment (Fig. 1A). This upregulation of *LeATL6* appeared to be a transient

Fig. 1. *LeATL6* expression in tomato roots treated with cell wall protein fraction (CWP) or the mycelial homogenate of *Pythium oligandrum*. **A**, Northern blot analysis of *LeATL6* and *PR6* expression in the roots of *Lycopersicon esculentum* cv. Micro-Tom and its *jai1-1* mutant treated with the CWP of *P. oligandrum* (CWP) and distilled water (DW) at 4 h after treatment. The expression of jasmonic acid (JA)-inducible *PR6* was analyzed to confirm the phenotype of the *jai1-1* mutant in which the JA-dependent signaling pathway was impaired. *rRNA* was detected as the internal RNA control. **B**, Time course of *LeATL6* expression in the roots of *L. esculentum* cv. Micro-Tom treated with the mycelial homogenate of *P. oligandrum* by Northern blot analysis. The transcript of *LeATL6* and *rRNA* as an internal control were analyzed 0, 2, 4, and 8 h after treatment with the mycelial homogenate containing CWP (MH) or DW.

response to CWP; this was because the induction of *LeATL6* expression was detected 2 h after treatment with the mycelial homogenate of *P. oligandrum* containing CWP, and then it gradually started decreasing 8 h after treatment (Fig. 1B). *LeATL6* expression also was induced in the CWP-treated *jai1-1* mutant tomato roots in which the JA-dependent signaling pathway was impaired. On the other hand, the expression of JA-inducible basic *PR6* encoding proteinase inhibitor II was not induced in *jai1-1* by CWP treatment (Fig. 1A). Therefore, we believe that *LeATL6* expression appears to be induced by CWP in tomato roots via a JA-independent signaling pathway or that *LeATL6* functions upstream of *JAI1-1* in the JA-dependent signaling pathway, which is activated by CWP.

Comparison of the amino acid sequences of *LeATL6* with other RING-H2 finger domain genes from plants.

The cDNA of *LeATL6* was cloned from the total RNA purified from CWP-treated tomato roots. The amino acid sequence deduced from *LeATL6* cDNA is shown in Figure 2. It was identical to the deduced amino acid sequence of the tomato expressed sequence tag clone (TIGR LeGI number TC126693) that was exhibited in the TIGR tomato genome database. Comparative analysis of the motif structure of other RING-H2 finger genes suggested that *LeATL6* contained a transmembrane-like hydrophobic region (region I), basic amino-acid-rich region (region II), a highly conserved amino acid sequence-containing region (region III), and a RING-H2 zinc-finger domain (region IV) flanked by acidic and basic amino acids, which commonly

are observed in the ATL family (Fig. 2) (Salinas-Mondragon et al. 1999). Further homology search of the amino acid sequence of *LeATL6* with those of the members of the ATL family indicated that *LeATL6* has the highest identity with *ATL6* of *A. thaliana* (*AtATL6*). Therefore, we believed *LeATL6* to be an ortholog of *AtATL6*.

Ubiquitin ligase activity of *LeATL6*.

As the first step toward analysis of whether *LeATL6* plays a functional role in CWP-induced defense responses, the ubiquitin ligase (E3) activity of *LeATL6* was measured; this was done because the ATL family proteins function as ubiquitin ligases in the ubiquitin/proteasome system (Serrano et al. 2006). Takai and associates (2002) have reported that they were able to get a sufficient amount of the fusion protein of maltose-binding protein (MBP) and truncated *EL5* that excluded the N-terminal transmembrane domain and the basic region by expression it in *Escherichia coli*. Therefore, to obtain a sufficient amount of active *LeATL6*, truncated *LeATL6* in which the N-terminal 98-amino acid region containing both the transmembrane domain and the basic region was deleted, and was expressed in *E. coli* as a fusion protein with MBP; the fusion protein was designated as MBP-*LeATL6* (Fig. 3A). Abundantly expressed MBP-*LeATL6* was present in the soluble protein fraction and was purified by amylose affinity chromatography (Fig. 3A). MBP-*LeATL6* was incubated with ubiquitin, ATP, and wheat germ lysate as a source of the ubiquitin-activating enzyme (E1) and the ubiquitin-conjugating enzyme (E2). Aliquots of the reaction mixture

L.esculentum <i>LeATL6</i>	1	-----MVNFKALSVNLLLVLLCPASQAQPSNSPDSENRFEYPSVSP-AMAI--IVILIAAL---FFIAFFSI	62
A.thaliana <i>ATL6</i>	1	MRSSDHMAFAGVLPVIFLLISSADLAASQSQPGPTNQPNYGRLLSP-AMAVI--VEILIAAL---FFRGFFSI	68
A.thaliana <i>ATL2</i>	1	-----MNSNDQDPIPRFPEDNNFSGSKTYAMSGKIM-LSAIVILFFVILMVFLHLYARWYL--	58
O.sativa <i>EL5</i>	1	-----MVRGVEQGGPAMDESSSSSPSPVAPAGQAAMTAGGIATVAIVLIVFAALTLA-FVLQCYCDER	65
I			
L.esculentum <i>LeATL6</i>	63	YIRNRGAVNGSIGQTF-SMRRRTAAATR-----GLEDSVIETFTFTTYEVKD	109
A.thaliana <i>ATL6</i>	69	NFRHCSGVPDAGVSPAGGVSRATVNAAAR-----GLDVSVVETFTFTLYSDVKD	118
A.thaliana <i>ATL2</i>	59	-----LRARRRHLLRRRSNRRATMVFFTAADPFTAAATSVVASRGLDPNVLSLPLVETESDETH	113
O.sativa <i>EL5</i>	66	RRAVTTTSTSG-----RGRPRPRRR-----SGSGDGGTGGGVDPVLRSLPVTVYSRSTA	117
II			
L.esculentum <i>LeATL6</i>	110	HH-----IGKGALECAVCLNEFEDDEMLRLTPKCDHVFHPECIDAWLKSHMTCPVCRADLTPKPDEPVPVQT	176
A.thaliana <i>ATL6</i>	119	QK-----LGKGELECATCLNEFEDDETLLRLPKCDHVFHPCIDAWLEAHMTCPVCRANLAQVAEGESVPE	185
A.thaliana <i>ATL2</i>	114	-----KDPIECVCLSEFESEETGRVLPNCOHTTFHVCIDVWFHSHSTCPLCRSLVESLAGIESTAAA	176
O.sativa <i>EL5</i>	118	AAAAKEEEEEDDDGVLECAVCLAELEDGEARFLRCGHGFHAECDVWLGSHSTCPLCRLTVVPPPPPLPPVPP	191
III			
L.esculentum <i>LeATL6</i>	177	PEVNRELQGEQENTQNNNEVTIQIDVDENLMSQGEETSVKPEVKRNLNFNAVNRPPRSFSLKRPKMLSKFRSHS	250
A.thaliana <i>ATL6</i>	186	GGTEPDLELQVVVNPEPVVTAAPVPEQLVTSEVDSRRLPGVPDLKRVKFSRSHTTGHSVVQPGECTERTLRL	259
A.thaliana <i>ATL2</i>	177	REREVIVAVSDPVLVIEPSSSSGLTDEPHGSGSSQMLREDSGRKPAAEVPRRTFSEFEDELTRRDSPASQSF	250
O.sativa <i>EL5</i>	192	EPPASYTVSLPASVLLGLSDHGAGAVTMTAEGRSTLVIEIPESAASTTPRDAAARSSPSLARLRLRLWSFGR	265
IV			
L.esculentum <i>LeATL6</i>	251	TGHSLVVPGENLDRLYLRLPEKVRKDVMDRALLNRTSCAVTLPRHGSTRGYRAGPGEKSNRGARSFKRIDRF	325
A.thaliana <i>ATL6</i>	260	PEDVRKRIMKDWKLNRTNSLLVLPGRGSSRRGKPIDRSRARSRLFRKTPSFLWRSRDDGSIRLGATGSVRAS	333
A.thaliana <i>ATL2</i>	251	RSPMSRMLSFRMLSRDRSASSPIAGAPPLSPTLSCTRIQMTESDIERGGEGR	304
O.sativa <i>EL5</i>	266	QGAAGSTSSCSCATGGDNDGDVEHGVSVTVAIRAVEAATPARPPEAEAGARTAAAHVRN	325
V			
L.esculentum <i>LeATL6</i>	326	DPETKSDRWFLKIAPFFTRGSSMKSPKVRADNEEASTSRNMKVAVKLPSFQCLEPKGDEPGLLANDSARSPV	398
A.thaliana <i>ATL6</i>	324	AVPNSTGSDSVRAGDRWAFRLNASFLWRNSSVHVPRGGVKNKGEGTSVKSTGASGSTSGSVRLPV	398
A.thaliana <i>ATL2</i>	304		304
O.sativa <i>EL5</i>	325		325

Fig. 2. Multiple alignment of the amino acid sequences of *LeATL6* and other really interesting new gene (RING)-H2 finger proteins. The amino acid sequences deduced from the cDNAs of *LeATL6*, *Arabidopsis ATL2*, and *ATL6* (GenBank accession numbers L76926 and AF132016, respectively) and rice *EL5* (AB045120) were aligned. The transmembrane-like hydrophobic region (I), the basic amino-acid-rich region (II), the highly conserved amino acid sequence-containing region (III), and the RING-H2 zinc finger domain (IV), which commonly are observed in the *ATL* gene family, are indicated with surrounding lines. Basic residues are shown against a gray background in region II. The amino acid residues that are common to regions I, III, and IV are shown against a black background.

were sampled and subjected to 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and MBP-LeATL6 then was detected immunologically using the anti-MBP antibody. Modified high molecular weight MBP-LeATL6 bands appeared after incubation of MBP-LeATL6 with the wheat germ lysate, whereas they did not appear in the absence of the wheat germ lysate (Fig. 3B). When MBP alone was incubated with ubiquitin, ATP, and the wheat germ lysate, high molecular weight bands were not observed (Fig. 3B). These results indicated that LeATL6 possessed E3 activity by which it catalyzed the transfer of ubiquitin to the MBP moiety. Although low molecular weight protein bands also were detected by anti-MBP antibody (Fig. 3B), they seemed to be a partial degradation product of full MBP-LeATL6 protein, because similar bands appeared in MBP-LeATL6 fusion protein fraction (Fig. 3A, lane 5A) purified by amylose affinity chromatography.

Coexpression of CaMV 35S promoter-controlled *LeATL6* and JA-dependent *PR6* and *TPI-1* promoter-controlled *DsRed* reporter constructs in tomato cells.

Using particle bombardment, tomato cells were introduced with a combination of *35Spro:LeATL6* and *35Spro:GFP* constructs in which *LeATL6* and *GFP* were under the control of the *Cauliflower mosaic virus* (CaMV) 35S promoter, respectively, or a single *35Spro:GFP* construct as a control with or without the *PR6pro:DsRed* construct in which the *DsRed* reporter was under the control of the JA-responsive basic *PR6* promoter. Initially, construct-introduced cells were identified by detecting GFP fluorescence followed by DsRed fluorescence caused by the activation of the *PR6* promoter in these cells (Fig. 4A and B). In the cells expressing *GFP*, approximately 10% of the cells exhibited *DsRed* expression without the *35Spro:LeATL6* construct (Fig. 5), whereas GFP fluorescence was noticeably detected in all cells expressing *DsRed* (data not shown).

When *LeATL6* was transiently overexpressed under the control of the CaMV 35S promoter in tomato cells, *DsRed* expression under the control of the *PR6* promoter was induced (Fig. 4A). The *DsRed* signal was observed in 31 of 60 cells that expressed GFP (Fig. 5A). However, when *PR6pro:DsRed* and *35Spro:GFP* constructs were coexpressed in tomato cells, a weak *DsRed* signal was observed in only 13 of the 60 cells that expressed GFP (Fig. 5A). This GFP signal also was observed at the emission range for the detection of the *DsRed* signal because this range enables the detection of the GFP signal (Fig. 4). This significant increase in the number of *DsRed*-expressing cells by the coexpression of *LeATL6* suggested that *PR6pro:DsRed* expression depended on the overexpression of *LeATL6* but was independent of *GFP* expression. The *DsRed* signal was not observed in any of the cells introduced with only a combination of the *35Spro:LeATL6* and *35Spro:GFP* constructs or with the single *35Spro:GFP* construct (Fig. 4A). When *35Spro:LeATL6* was coexpressed with the *NtPR2pro:-DsRed* construct in which *DsRed* expression is under the control of the SA-responsive acidic *PR2* promoter, a weak *DsRed* signal was observed in only 13 of the 60 cells that expressed GFP. This implied that the acidic *PR2* promoter was not activated by the overexpression of *LeATL6* (Figs. 4A and 5A).

To further confirm the activation of basic defense-related gene expression by overexpression of *LeATL6*, expression of a second set of defense-related genes, basic proteinase inhibitor I (*TPI-1*) and acidic *PR-1* genes, was examined with or without *35Spro:LeATL6* construct. When *TPI-1pro:DsRed* was coexpressed with the *35Spro:LeATL6* construct, the *DsRed* signal was observed in 28 of 60 cells that expressed GFP (Figs. 4C and 5B). However, when the *NtPR1pro:DsRed* was coexpressed with the *35Spro:LeATL6* construct, a weak *DsRed* signal was observed in only 14 of the 60 cells that expressed GFP.

These results indicated that *LeATL6* overexpression activated the basic *PR6* and *TPI-1* promoters but not the acidic *PR2* and *PR1* promoters in wild tomato cells.

When a combination of the *35Spro:LeATL6* and *35Spro:GFP* constructs was introduced into *jail-1* mutant tomato cells with the *PR6pro:DsRed* construct, the DsRed fluorescence signal was observed in only approximately 10 of the 60 DsRed-expressing cells of the *jail-1* mutant as well as in the *jail-1* mutant tomato cells introduced with the *35Spro:GFP* and *PR6pro:DsRed* constructs (Figs. 4B and 5A). The expression of *TPI-1pro:DsRed* by overexpression of *35Spro:LeATL6* also was cancelled in the *jail-1* (Figs. 4D and 5B). These results indicated that the activation of the basic *PR6* and *TPI-1* promoters by *LeATL6* overexpression probably is induced in a *JAIL-1*-dependent manner.

DISCUSSION

Arabidopsis spp. have approximately 470 genes that encode RING zinc-finger domain proteins. The *ATL* gene family that encodes putative RING-H2 finger-type E3 ubiquitin ligases and comprises 80 members is one class of these 470 genes (Jensen et al. 1998; Serrano et al. 2006). The expression of

Fig. 3. Expression of the maltose-binding protein (MBP)-LeATL6 fusion protein in *Escherichia coli* and detection of ubiquitination activity. **A**, Truncated LeATL6, in which the N-terminal 89-amino-acid region was deleted, was expressed in *E. coli* as a fusion protein with MBP. *E. coli* strain BL21 containing pMAL-p2:LeATL6 in which the truncated *LeATL6* cDNA was cloned downstream of MBP in the frame (A) or only pMAL-p2 as a control (C) was cultured without (1) or with (2–5) IPTG. Total protein in the cell extract (1 and 2), crude protein extract (3), and insoluble protein fraction (4) as well as MBP-LeATL6 and MBP that were purified by amylose affinity chromatography (5) were separated by 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis. **B**, MBP-LeATL6 was modified when incubated with wheat germ lysate as a source of E1 and E2. Aliquots of the reaction mixture containing MBP-LeATL6, ubiquitin, ATP, and wheat germ lysate were sampled at 0, 5, 15, 30, 60, 120, 180, and 240 min after incubation (1). As a control, the reaction mixture lacking wheat germ lysate (2) and containing MBP instead of MBP-LeATL6 (3) was incubated for 240 min. These reaction mixtures were subjected to immunoblotting with the anti-MBP antibody.

some members of the *ATL* gene family in response to elicitors also has been characterized in *A. thaliana* and *Oryza sativa* (Salinas-Mondragon et al. 1999; Serrano et al. 2006; Takai et al. 2001). The expression of *ATL2* and *ATL6* in *A. thaliana*

was induced 15 to 30 min after treatment with chitin or cellulysin (Salinas-Mondragon et al. 1999). The *EL5* gene of *O. sativa*, which shows structural similarity to the *ATL* genes of *A. thaliana*, also was upregulated 45 min after treatment with an

N-acetylchitoooligosaccharide elicitor (Takai et al. 2001). In our experiments, the induction of the expression of *LeATL6*, an ortholog of *ATL6*, in early response to a fungal elicitor in tomato also was demonstrated. Therefore, early elicitor induction of a member of the *ATL* gene family and its orthologous genes encoding the RING-H2 finger protein may be a general response in monocot and dicot plant species. However, we need to study the downstream responses that are controlled by defense regulatory protein targeted by ubiquitination with RING-type E3 ubiquitin ligases encoded on some members of *ATL* gene family (e.g., *ATL2* and *ATL6*).

It is well known that an elicitor induces a set of defense responses: accumulation of PR proteins, production of reactive oxygen species, ion fluxes, and phytoalexin synthesis (Boller 1995). Thus far, the relationship between the upregulation of elicitor-responsive *ATL* genes and the activation of these defense responses was not elucidated due to limited understanding. However, our result suggested that *LeATL6* expression under the control of the CaMV 35S promoter activated the basic *PR6* and *TPI-1* promoters in wild tomato, indicating that elicitor-induced *LeATL6* expression might regulate the activation of certain members of the *PR* gene family and some other defense responses. Basic *PR6* and *TPI-1*, encoding proteinase inhibitor II and I, respectively, generally are regulated via JA-dependent signaling pathway (Glazebrook 2001; Li et al. 2004). In fact, the *PR6* transcript did not accumulate in the CWP-treated *jail-1* mutant tomato plants. In our previous experiment, the CWP-induced resistance in tomato appeared to activate the JA- and ethylene-dependent signaling pathways (Hase et al. 2006; Takahashi et al. 2006). Furthermore, CWP-treated wild tomato showed enhanced resistance to *Ralstonia solanacearum*, whereas the CWP-induced resistance was compromised in JA-insensitive *jail-1* mutant tomato (S. Hase, A. Shimizu, K. Nakaho, S. Takenaka, and H. Takahashi, unpublished results). Therefore, *LeATL6* probably plays an important role in the CWP-induced resistance to pathogens via JA-dependent signaling pathways.

More direct evidence of the regulation of the defense response by *LeATL6* may be obtained by studying transgenic tomato overexpressing *LeATL6* under the control of the CaMV 35S promoter. However, although we attempted to stably introduce the *35Spro:LeATL6* construct into tomato by using an *Agrobacterium*-mediated transformation system, all transgenic plants that overexpressed *LeATL6* died during regeneration of the transformants. It has been demonstrated that yeast cells that express *Arabidopsis ATL2* have a lethal phenotype (Martinez-Garcia et al. 1996). *Arabidopsis* mutants in which the expression of the members of the *ATL* gene family was constitutively induced showed a severely stunted phenotype (Serrano and Guzman 2004). Furthermore, the overexpression of the alfalfa *MsRH2-1* gene that shows the greatest homology to *ATL4* induces pleiotropic effects on plant growth and development (Karlowski and Hirsch 2003). These facts suggest that it may be difficult to obtain transgenic tomato plants overex-

pressing *LeATL6*. Therefore, in our experiment, *LeATL6* was expressed transiently to analyze the relationship between the upregulation of *LeATL6* and *PR* gene expression. A weak DsRed signal was observed in some cells introduced with the *PR6pro:DsRed* and *35Spro:GFP* constructs (Fig. 5); however, this phenomenon appears to be caused by mechanical wounding due to particle bombardment. This is because the induction of *PR6* and *TPI-1* gene expression by mechanical wounding has been reported frequently in tomato (Ryan 2000). On the other hand, on introducing the *PR6pro:DsRed* and *35Spro:GFP* constructs, the number of cells that expressed DsRed in tomato cells with *35Spro:LeATL6* was threefold higher than that in tomato cells without *35Spro:LeATL6*. This significant increase in the number of cells expressing DsRed by the coexpression of *35Spro:LeATL6* indicates that the transient assay method using particle bombardment is effective for detecting *LeATL6*-regulated *PR6* gene expression. The same transient assay method also was proved by detecting *LeATL6*-regulated *TPI-1* gene expression.

The role of ubiquitin ligase E3 has been studied more extensively for its gene-for-gene resistance with respect to *Avr-R* gene interaction (Durrant et al. 2000; Gonzalez-Lamothé et al. 2006; Kawasaki et al. 2005; Yang et al. 2006), although the association of the ubiquitin/proteasome system with plant-pathogen interaction also was suggested in tobacco plants systemically infected with *Tobacco mosaic virus* and in cucumber seedlings treated with specific inhibitors of proteasome activity (Becker et al. 2000; Takizawa et al. 2005). *P. oligandrum* has been utilized as a biocontrol agent for controlling the damping-off of cress caused by *P. ultimum* and has been commercialized as Polygardron (Polyversum) in the Slovak Republic (Brozova 2002; Butt and Copping 2000). The glycoprotein elicitor CWP, which is present in the cell wall fraction of *P. oligandrum*, appears to play an important role in enhancing the potency of *P. oligandrum* in controlling plant diseases. This is based on the fact that the damping-off disease caused by *Rhizoctonia solani* AG2-2 in sugar beet, Fusarium head blight caused by *Fusarium graminearum* in wheat, and bacterial wilt disease caused by *R. solanacearum* in tomato were suppressed by the treatment with CWP (Hase et al. 2006; Takenaka et al. 2003). Furthermore, induction of defense-related gene expression by CWP treatment also has been observed in sugar beet, rice, and potato (Takenaka et al. 2006) (A. Shimizu and H. Takahashi, unpublished results). CWP possesses two major proteins, POD-1 and POD-2 (Takenaka et al. 2006). POD-1 and POD-2 contain elicitin signatures and O-linked glycosylation sites which may be associated with its linkage to the cell wall of *P. oligandrum*, and it is easily released in vitro from the cell wall at high CaCl₂ concentrations (Takenaka et al. 2003). The general elicitor activity and indispensable structure of POD for the microbial lifestyle suggest that CWP is recognized as an invariant PAMPs by the plant surveillance system. The finding that the induction of *LeATL6* by CWP treatment regulates the expression of JA-dependent

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Fig. 4. Transient *DsRed* expression under the control of the basic *PR6* and *TPI-1* promoters by overexpression of *LeATL6* in the wild and *jail-1* mutant tomato cells. **A**, A series of the combination of the *35Spro:LeATL6*, *LePR6pro:DsRed*, *NtPR2pro:DsRed*, and *35Spro:GFP* constructs was introduced into the epidermal cells of wild tomato leaves by the particle bombardment. **B**, A combination of the *35Spro:LeATL6*, *LePR6pro:DsRed*, and *35Spro:GFP* constructs and that of *LePR6pro:DsRed* and *35Spro:GFP* were introduced into the epidermal cells of wild and *jail-1* mutant tomato leaves by the particle bombardment. **C**, A series of the combination of the *35Spro:LeATL6*, *TPI-1pro:DsRed*, *NtPR1pro:DsRed*, and *35Spro:GFP* constructs was introduced into the epidermal cells of wild tomato leaves by the particle bombardment. **D**, A combination of the *35Spro:LeATL6*, *TPI-1pro:DsRed*, and *35Spro:GFP* constructs and that of *TPI-1pro:DsRed* and *35Spro:GFP* were introduced into the epidermal cells of wild and *jail-1* mutant tomato leaves by the particle bombardment. In both experiments, the leaves were observed 12 h after bombardment by using a fluorescence microscope. The GFP signal was detected using Zwiss filter no. 10 (excitation at 450 to 490 nm per emission at 515 to 565 nm), whereas the DsRed signal was detected using Zwiss filter no. 15 (excitation at BP546 nm per emission at LP590 nm). Filter no. 15 could not exclude the GFP signal; therefore, a yellow-red-colored signal that was caused by the overlap of the fluorescence of DsRed and GFP was observed using filter no. 15.

basic *PR-6* will provide a better understanding of the role of ubiquitin ligase E3 in PAMP-mediated defense in plants.

The MBP-LeATL6 fusion protein produced in *E. coli* exhibited ubiquitin ligase activity in vitro when incubated with ubiquitin, ATP, and wheat germ lysate as a source of the ubiquitin-activating enzyme (E1) and ubiquitin-conjugated enzyme (E2). *Arabidopsis* spp. possess two genes corresponding to E1 isoforms and approximately 45 genes corresponding to E2 or E2-like proteins; however, almost 1,200 genes appear to encode E3 enzymes (Smalle and Vierstra 2004). However, certain E1 and E2 proteins that should interact with LeATL6 as E3 in vivo have not been identified. Among the three enzymes, E3 plays a major role in determining the specificity of a substrate for ubiquitination. Although the target protein for LeATL6 has not been identified, the C-terminal half of the protein belonging to the ATL family that contains a variable region appears to

determine the target specificity. Therefore, the identification of E1 and E2 proteins that function with LeATL6 and the characterization of the target protein of LeATL6 will provide novel insights on the downstream signaling mechanisms of LeATL6 in PAMP-induced defense responses.

MATERIALS AND METHODS

Plants and fungal cultures.

Lycopersicon esculentum cv. Micro-Tom and its *jai1-1* mutant (Li et al. 2004) were grown in quartz sand at 24°C in a growth chamber under continuous fluorescent light (70 $\mu\text{mol m}^{-2} \text{s}^{-1}$) and fertilized with a thousand-fold diluted Hyponex solution (Hyponex Japan, Osaka, Japan) at 3-day intervals (Takahashi et al. 2005). The *P. oligandrum* (PO) isolate MMR2 was cultured in 10% V8 broth containing 0.15 M

Fig. 5. Number of cells expressing the *DsRed* under the control of the basic *PR6* and *TPI-1* promoters by overexpression of *LeATL6* in the wild and *jai1-1* mutant tomato cells. **A**, Number of cells expressing the *DsRed* fluorescence signals when a series of a combination of the *35Spro::LeATL6*, *LePR6pro::DsRed*, *NtPR2pro::DsRed*, and *35Spro::GFP* constructs were introduced into wild and *jai1-1* mutant tomato cells. The number of *DsRed*-expressing cells per 60 GFP-expressing cells was counted. Average number of *DsRed*-expressing cells in the triplicate experiments was shown. The bars indicate standard deviation (SD) for the triplicate experiments. Asterisks indicate a significant difference between number of the cells expressing the *DsRed* fluorescence signal in a combination of the *35Spro::LeATL6*, *LePR6pro::DsRed*, and *35Spro::GFP* constructs compared with other combinations of vector constructs according to a *t* test ($P < 0.05$). **B**, Number of cells expressing the *DsRed* fluorescence signals when a series of a combination of the *35Spro::LeATL6*, *TPI-1pro::DsRed*, *NtPR1pro::DsRed*, and *35Spro::GFP* constructs were introduced into wild and *jai1-1* mutant tomato cells. The number of *DsRed*-expressing cells per 60 GFP-expressing cells was counted. Average number of *DsRed*-expressing cells in the triplicate experiments was shown. The bars indicate SD for the triplicate experiments. Asterisks indicate a significant difference between number of the cells expressing the *DsRed* fluorescence signal in a combination of the *35Spro::LeATL6*, *TPI-1pro::DsRed*, and *35Spro::GFP* constructs compared with other combinations of vector constructs according to a *t* test ($P < 0.05$).

CaCO₃ and 0.1% of wheat germ oil at 25°C for 3 weeks. The mycelial mats were collected by filtration and washed with distilled water.

Treatment with the mycelial homogenate of *P. oligandrum*.

Fourteen-day-old tomato plants were harvested carefully from the plants grown in quartz sand; care was taken to minimize damage to the root tissue by preventing injury. After rinsing the roots three times with distilled water, the roots of one plant were dipped into 1 ml of mycelial homogenate containing 5×10^5 oospores and incubated at 24°C under continuous fluorescent light ($70 \mu\text{mol m}^{-2} \text{s}^{-1}$). The mycelial homogenate was prepared as described previously (Hase et al. 2006).

Cloning of *ATL6* cDNA from tomato.

Total RNA was isolated from CWP-treated tomato roots by using an RNeasy plant mini kit (Qiagen, Hilden, Germany). Five micrograms of the total RNA was reverse transcribed to synthesize first-strand cDNA by using an oligo(dT) primer and the ThermoScript reverse-transcription polymerase chain reaction (PCR) system (Invitrogen, Carlsbad, CA, U.S.A.). Using the first-strand cDNA as a template, *LeATL6* cDNA was amplified by PCR. For the PCR, 1 μg of the first-strand cDNA was added to 50 μl of 10 mM Tris-HCl (pH 8.3) containing 50 mM KCl; 2 mM MgCl₂; 0.2 mM each dATP, dCTP, dGTP, and dTTP; 5 units of KOD-plus DNA polymerase (Toyobo, Kyoto, Japan); and 0.2 μM of primers *LeATL6*-F1 (5'-CCACCAAAA GCTGCCATGG-3') and *LeATL6*-R1 (5'-ATTCTTTAAACCG GAGATCG-3'). PCR was performed as follows: 30 cycles at 94°C for 1 min, 55°C for 2 min, and 73°C for 1 min. The PCR product was purified and then cloned into the *EcoRV* site of pBluescript SK+ (Stratagene, La Jolla, CA, U.S.A.) by the procedure described previously (Takahashi and Ehara 1993). To confirm that the DNA of interest had been cloned, the nucleotide sequence of the insert was determined by the Sanger method using the automated DNA sequencer ABI model 310A (Applied Biosystems, Foster City, CA, U.S.A.), and the resultant plasmid vector was named pBS+SK.*LeATL6*.

Northern hybridization analysis.

Total RNA was isolated from the roots of the tomato plants by using an RNeasy plant mini kit according to the instruction manual. Ten micrograms of total RNA was loaded in each lane of a 1.2% denaturing agarose gel. Northern hybridization was performed according to a published protocol (Sambrook and Russell 2001). To detect *LeATL6* expression, cDNA was labeled with [³²P]dCTP by using the Megaprime DNA labeling systems (Amersham-Pharmacia, Piscataway, NJ, U.S.A.).

Expression of the *ATL6* protein in *E. coli*.

By using PCR, we amplified the *LeATL6* fragment encoding a truncated *LeATL6* protein that lacked N-terminal 89 amino acids and contained a highly conserved amino acid sequence-containing region (region III) and a RING-H2 zinc-finger domain (region IV) (Fig. 2). pBS+SK.*LeATL6* served as a template and the two primers *LeATL6*-ERI-F1 (5'-GGAATTCGG ACTTGAAGATTCCGTCATCG-3') and *LeATL6*-ERI-R1 (5'-GGAATTCATTCTTTAAACCGGAGATCG-3') were used (the *EcoRI* sites are underlined). The PCR conditions were similar to those described above. The PCR product was purified, digested with *EcoRI*, and then cloned into the *EcoRI* site of pMAL-p2X (New England BioLabs, Beverly, MA, U.S.A.). The resultant plasmid was designated pMAL-p2X:*LeATL6*ΔN. In order to avoid the synthesis of a fusion protein of MBP and a partial fragment of *lacZα* located downstream of MBP in the pMAL-p2X frame in *E. coli*, *EcoRI*-digested pMAL-p2X DNA was blunted by treatment with T4 DNA polymerase and

then religated, thereby causing a shift in the open reading frame of *lacZα*. pMAL-p2X:*LeATL6*ΔN and the obtained pMAL-p2 (control) were transformed into *E. coli* strain BL21. IPTG-inducible production of the recombinant protein and its purification by amylose affinity chromatography were performed according to the manufacturer's instructions.

Ubiquitin ligase activity assay.

The in vitro ubiquitination experiment was performed as described by Takai and associates (2002). The reaction mixture was incubated at 30°C for 0, 5, 15, 30, 60, 120, 180, and 240 min, and the reaction was terminated by the addition of an equal volume of SDS-PAGE buffer (Laemmli 1970). After boiling for 5 min, the reaction mixtures were loaded on a 7.5% SDS-PAGE gel and electrophorized. MBP-*LeATL6* was detected immunologically using the anti-MBP antibody (New England BioLabs).

Transient assay of the activity of the basic *PR6* and *TPI-1* promoters and the acidic *PR1* and *PR2* promoters under coexpression of *LeATL6* in tomato cells.

The promoter fragments of tomato basic *PR6* encoding proteinase inhibitor II (Zhang et al. 2004), tomato basic *TPI-1* encoding proteinase inhibitor I (*TPI-1*) (Lee et al. 1986), tobacco acidic *PR2* encoding β -1,3-glucanase (Hennig et al. 1993), and tobacco acidic *PR1* (Grüner and Pfitzner 1994) were amplified by PCR from the genomic DNA of tomato and tobacco. The template DNA for PCR was isolated using the cetyltrimethylammonium bromide method (Murray and Thompson 1980). One microgram of genomic DNA was added to 50 μl of 10 mM Tris-HCl (pH 8.3) containing 50 mM KCl; 2 mM MgCl₂; 0.2 mM each dATP, dCTP, dGTP, and dTTP; 5 units of KOD-plus DNA polymerase (Toyobo); and 0.2 μM of the primers *LePR6b.pro-F1* (5'-CCCAAGCTTGGGATTTTAATA ATTGTATAACG-3', in which the *HindIII* site has been underlined) and *LePR6b.pro-R1* (5'-CATGCCATGGTGGATAATT GATTAC-3', in which the *NcoI* site has been underlined) for basic *PR6*, *TPI-1pro-F1* (5'-CCCAAGCTTGAATTCACCTCA AAACATAA-3', in which the *HindIII* site has been underlined) and *TPI-1pro-R1* (5'-CATGCCATGGTGTGTTTAGTTG TAAAGAGT-3', in which the *NcoI* site has been underlined) for basic *TPI-1*, the primers *NtPR2a.pro-F1* (5'-CCCAAGCTT GGGGAATTCTG-AACTTAGTTATG-3', in which the *HindIII*-digestion site has been underlined) and *NtPR2a.pro-R1* (5'-CATGCCATGGCATGAGCTAAAATGGAAATGAGAG-3', in which the *NcoI* site has been underlined) for acidic *PR2*, or *NtPR1apro-F1* (5'-CCCAAGCTTGATCGTAGCACATAAAC GTT-3', in which the *HindIII* site has been underlined) and *NtPR1apro-R1* (5'-CATGCCATGGCTATAGGAGAAATGTT GTA-3', in which the *NcoI* site has been underlined) for acidic *PR-1*. The PCR conditions were identified to those described earlier. The resultant DNA fragment was purified, digested with *HindIII* and *NcoI*, and then cloned between the *HindIII* and *NcoI* sites of Impact vector 1.1 (Plant Research International B. V., Wageningen, The Netherlands). The resultant plasmid constructs were designated pLePR6pro:rbcsT, pTPI-1pro:rbcsT, pNtPR2pro:rbcsT, and pNtPR1pro:rbcsT, respectively. In these constructs, the *rbcs* terminator fragment was located downstream of the promoters. To monitor the promoter activity, the *Bam*HI- and *NorI*-digested *DsRed* fragment that was amplified from pDsRed2 (Clontech-TAKARA, Kyoto, Japan) by PCR using the two primers *DsRed-F1* (5'-ACTCTA GAGGATCCCCGGGTACCGG-3', in which the *Bam*HI site has been underlined) and *DsRed-R1* (5'-TAAAGCGGCCGCC AGGACAGGTGGTGGCG-3', in which the *NorI* site has been underlined), by following the abovementioned procedure, was cloned between the *PR6*, *TPI-1*, *PR2*, or *PR1* promoter and the

rbcs terminator of *pLePR6pro:rbcsT*, *pTPI-1pro:rbcsT*, *pNtPR2pro:rbcsT*, and *pNtPR1pro:rbcsT*. The nucleotide sequence of the insert was determined, and the resultant plasmid vector constructs were named *LePR6pro:DsRed*, *pTPI-1pro:DsRed*, *pNtPR2pro:DsRed*, and *pNtPR1pro:DsRed*, respectively.

To develop a vector construct for the overexpression of *LeATL6* in tomato cells, the *LeATL6* fragment digested from *pBS+SK.LeATL6* was cloned between the *CaMV 35S* promoter and the *NOS* terminator of *p35S50KP-GFP* (Satoh et al. 2000) by substituting it for the *Bam*HI- and *Sac*I-digested 50KP-GFP fragment in *p35S50KP-GFP*. The resultant plasmid construct was named *35Spro:LeATL6*. The *35Spro:GFP* construct was generated by the deletion of the *Bam*HI- and *Sac*II-digested 50KP fragment from *p35S50KP-GFP*.

To detect the *LeATL6*-dependent activation of the basic *PR6*, basic *TPI-1*, acidic *PR2*, and acidic *PR1* promoters, a series of a combination of the *35Spro:LeATL6* and *LePR6pro:DsRed*, *pTPI-1pro:DsRed*, *pNtPR2pro:DsRed*, or *pNtPR1pro:DsRed* constructs was introduced into the epidermal cells of tomato leaves by particle bombardment (PDS-1000/He particle delivery system; Bio-Rad, Hercules, CA, U.S.A.) with the *35Spro:GFP* construct which was coexpressed for the identification of the bombarded cells. The leaves then were incubated at 25°C for 12 h under continuous fluorescent light (70 $\mu\text{mol m}^{-2} \text{s}^{-1}$). Under a fluorescence microscope (Zwiss Axioplan 1, Oberkochen, Germany), GFP fluorescence was detected using Zwiss filter no. 10 (excitation at 450 to 490 nm per emission at 515 to 565 nm) and DsRed fluorescence, using filter no. 15 (excitation at BP546 nm per emission at LP590 nm). The degree of *LeATL6*-dependent activation of the basic *PR6*, basic *TPI-1*, acidic *PR2*, and acidic *PR1* promoters was determined in wild and *jail-1* mutant tomato by counting the number of DsRed-expressing cells per 60 GFP-expressing cells.

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AUTHOR-RECOMMENDED INTERNET RESOURCE

The Institute for Genomic Research (TIGR) tomato genome database: www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=tomato