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A Novel Plant Cysteine Protease Has a Dual Function as a Regulator of 1-Aminocyclopropane-1-Carboxylic Acid Synthase Gene Expression [W]

Noa Matarasso, Silvia Schuster, and Adi Avni[1]

Department of Plant Sciences, Tel Aviv University, Tel Aviv 69978, Israel

The hormone ethylene influences plant growth, development, and some defense responses. The fungal elicitor Ethylene-Inducing Xylanase (EIX) elicits ethylene biosynthesis in tomato (*Lycopersicon esculentum*) and tobacco (*Nicotiana tabacum*) leaves by induction of 1-aminocyclopropane-1-caboxylic acid synthase (*Acs*) gene expression. A minimal promoter element in the *LeAcs2* gene required for EIX responsiveness was defined by deletion analysis in transgenic tomato plants. The sequence between −715 and −675 of the tomato *Acs2* gene was found to be essential for induction by EIX. A Cys protease (LeCp) was isolated that specifically binds to this *cis* element in vitro. Ectopic expression of LeCp in tomato leaves induced the expression of *Acs2*. Moreover, chromatin immunoprecipitation showed that LeCp binds in vivo to the *Acs* promoter. We propose a mechanism for the dual function of the LeCp protein. The protease acts enzymatically in the cytoplasm. Then, upon signaling, a small ubiquitin-related modifier protein binds to it, enabling entrance into the nucleus, where it acts as a transcription factor. Thus, LeCp can be considered a dual-function protein, having enzymatic activity and, upon elicitor signaling, exhibiting transcriptional factor activity that induces *LeAcs2* expression.

INTRODUCTION

Ethylene as a plant hormone influences many aspects of plant growth and development (Mattoo and Suttle, 1991; Abeles et al., 1992; Bleecker and Kende, 2000; Mattoo and Handa, 2003) as well as the induction of some plant defense responses (Boller, 1991; Eyal et al., 1993; Lund et al., 1998; Ciardi et al., 2000). The biosynthetic pathway of ethylene production in higher plants is well defined (Yang and Hoffmann, 1984). 1-Aminocyclopropane-1-carboxylic acid (ACC) synthase is a cytosolic enzyme that catalyzes the first committed step in ethylene biosynthesis in higher plants. It is a key regulatory enzyme that catalyzes the production of the ethylene precursor ACC from *S*-adenosylmethionine. ACC synthase (*Acs*) cDNAs and genomic sequences have been cloned from numerous plant species (Zarembinski and Theologis, 1994; Alexander and Grierson, 2002). The picture emerging from the study of these genes is that ACC synthase is encoded by members of a divergent multigene family in which each gene is differentially regulated by various environmental and developmental factors during the growth of a plant. *Arabidopsis thaliana*, for example, has nine *Acs* genes and tomato has at least eight gene family members, which are differentially regulated by inducers such as auxin, fruit ripening, elicitors, and wounding (Olson et al., 1991; Rottmann et al., 1991; Lincoln et al., 1993; Avni et al., 1994; Abel et al., 1995; Ge et al., 2000; Yamagami et al., 2003).

Ethylene-Inducing Xylanase (EIX), a 22-kD protein elicitor (Dean and Anderson, 1991), elicits ethylene biosynthesis, hypersensitive response, and other defense responses in such plants as tomato (*Lycopersicon esculentum*) and tobacco (*Nicotiana tabacum*) (Bailey et al., 1992; Felix et al., 1993; Avni et al., 1994; Yano et al., 1998; Elbaz et al., 2002; Ron and Avni, 2004). The increase in ethylene biosynthesis is accompanied by the accumulation of 1-aminocyclopropane-1-carboxylic acid (ACC), an increase in extractable ACC synthase activity, and increases in *Acs* and ACC oxidase transcripts (Avni et al., 1994). Data presented by us and others (Sharon et al., 1993; Enkerli et al., 1999; Furman-Matarasso et al., 1999; Rotblat et al., 2002) indicate that the enzymatic xylanase activity of EIX is unrelated to the elicitation process.

Programmed cell death, or apoptosis, is the mechanism by which animal cells activate an intrinsic suicide program to kill themselves (Vaux and Korsmeyer, 1999). Cys proteases have emerged as key enzymes in the regulation of animal programmed cell death. In plants, Cys proteases have been shown to function in many aspects of cellular regulation (Estelle, 2001; Elbaz et al., 2002). Expression of a Cys protease was shown to coincide with several developmental events associated with programmed cell death (Xu and Chye, 1999). The tomato Rcr3, which encodes a Cys protease, was shown to be a positive regulator of Cf-2–dependent resistance (Kruger et al., 2002). During citrus fruit ripening, a Cys protease was found to be induced (Alonso and Granell, 1995). An additional Cys protease was shown to be expressed during senescence and wounding (Kinoshita et al., 1999). In tomato, a Cys protease was found to

[1] To whom correspondence should be addressed. E-mail lpavni@post.tau.ac.il; fax 972-3-641-0195.

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accumulate in the leaves of drought-stressed plants. This protein is localized mainly in the nucleus and the chloroplasts of drought-stressed cells (Harrak et al., 2001). Vacuolar processing enzyme (VPE) is a Cys protease that exhibits substrate specificity toward Asp residues (Hara-Nishimura et al., 1993, 1998). The VPE was shown to be induced during wounding (Kinoshita et al., 1999; Yamada et al., 2004). Moreover, VPE exhibits a caspase-1 activity involved in *Tobacco mosaic virus*–induced hypersensitive cell death (Hatsugai et al., 2004).

Transcription factors are sequence-specific DNA binding proteins that are capable of activating and/or repressing transcription. They are largely responsible for the selectivity in gene regulation and are often expressed in a tissue-specific, developmental, or stimulus-specific manner. In Arabidopsis, there are ~3000 genes estimated to be involved in different aspects of transcription regulation (Arabidopsis Genome Initiative, 2000). These include many new factors with unknown roles. The Drosophila transcription factor Eyes absent was shown to belong to the phosphatase subgroup of the haloacid dehalogenases with Tyr phosphatase activity (Tootle et al., 2003). Recently, enzymes involved in Arg biosynthesis were shown to directly bind DNA in vivo (Hall et al., 2004). These results indicate that metabolic enzymes can directly regulate gene expression (Hall et al., 2004).

In this article, we describe the identification of a *cis* element in the promoter of tomato *Acs2* that regulates the expression of the *Acs2* gene when induced by the EIX elicitor. Furthermore, we describe the isolation and characterization of a Cys protease protein from tomato that binds in vitro and in vivo to the *cis* element in the *Acs* promoter and activates *Acs* gene expression.

RESULTS

Analysis of Acs2 Promoter Activity in Tomato Leaves Using the β-Glucuronidase Reporter System

Tomato ACC synthase is encoded by a multigene family in which each gene is differentially regulated. Gene-specific probes were generated to identify a member of the *Acs* gene family that has been induced by the EIX elicitor. We found that tomato *Acs2* is highly induced by EIX treatment (see Supplemental Figure 1 online). Our results agree with previous data showing that *Acs2* is induced by wounding, ripening, floral senescence, and a *Phytophthora infestans* elicitor (Rottmann et al., 1991; Lincoln et al., 1993; Spanu et al., 1994; Olson et al., 1995).

We used the β-glucuronidase (GUS) reporter gene to identify *cis*-acting elements involved in controlling the induction of *Acs2* by the EIX elicitor. The GUS open reading frame (ORF) was inserted downstream of the *Acs2* 3058-bp promoter in the *Bam*HI site. Site-directed promoter deletions at −807, −770, −715, −675, and −330 were generated (Figure 1A). A series of transgenic tomato plants harboring the full-length 3058 bp and the different deleted promoters were generated and analyzed for their responsiveness to the EIX elicitor. For each construct, the analysis was performed three times on 5 to 13 independent transformants. GUS activity was monitored in transgenic tomato leaves inoculated with water or EIX harvested 4 h after treatment. Compared with water treatment, EIX induced GUS activity in transgenic plants harboring the −3058 promoter and the −807, −770, and −715 deleted promoters but failed to induce GUS activity in transgenic plants harboring the −675 and −330 deletions (Figure 1B). We made a 40-bp deletion (−715 to −675) in the 3058-bp *Acs2* promoter and generated transgenic tomato plants harboring the deleted promoter upstream of the GUS reporter gene to test whether this sequence is essential for induction by EIX. EIX did not induce GUS activity in transgenic plants lacking the sequences between −675 and −715 in the 3058-bp promoter (Δ40 GUS; Figure 1B). EIX-treated −675-GUS, −330-GUS, and Δ40-GUS plants exhibited GUS activity in the range of the background, leading to the conclusion that the 40-bp fragment (−715 to −675) in the promoter sequence is essential for induction of *Acs2* by the EIX elicitor. A search of the database revealed that the 40-bp sequence

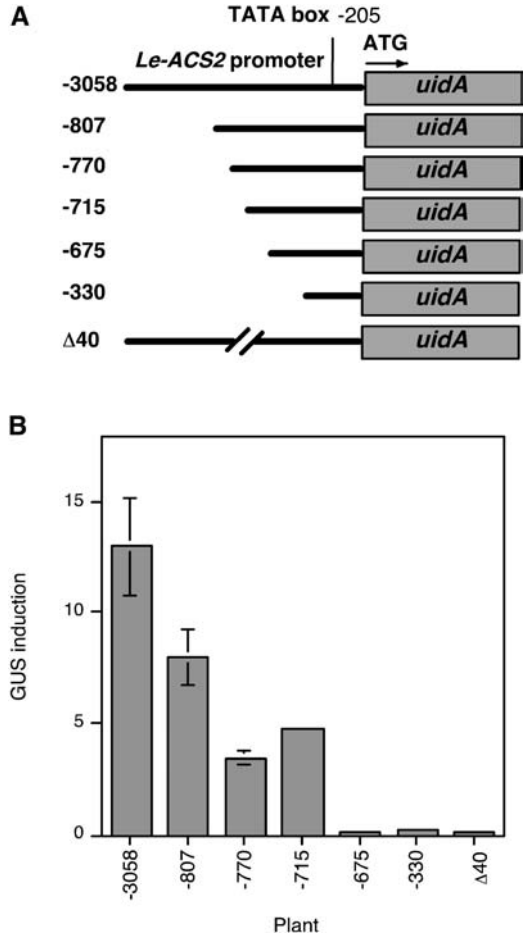

Figure 1. Induction of GUS Activity in Transgenic Tomato Harboring the Deleted Acs2 Promoter.

(A) Scheme of the different promoter deletions. The 3058-bp promoter and the deleted promoter fragments were cloned upstream of the *uidA* reporter gene (GUS).

(B) Transgenic plants harboring the different promoter deletions as indicated were treated for 4 h with EIX (1 μg/mL) elicitor. The GUS induction ratio (treated/untreated) is shown. The data in the graph correspond to the mean ± SE of three independent experiments.

contains the motif TAAAAT present in the promoter of the tobacco *HSR203J* gene, a molecular marker of the hypersensitive response (Pontier et al., 2001), and in the promoter of the Arabidopsis *AIG2* gene, which has been shown to be involved in defense responses (Reuber and Ausubel, 1996).

DNA–Protein Interaction with the Promoter of *Acs2*

Transcriptional regulation of eukaryotic genes is mostly triggered by *trans*-acting protein factors that bind to specific *cis*-acting sequences in the promoter (Dynan and Tjian, 1985). Potential DNA–protein interactions in the promoter region of *Acs2* were analyzed by gel-shift assays. Nuclear proteins extracted from tobacco leaves of EIX-treated plants were reacted with the 40-bp fragment (−715 to −675) from the *Acs2* promoter. As shown in Figure 2, the 40-bp fragment forms a specific complex with nuclear extracts. The specificity of the complex was monitored by competition with 10- and 30-fold molar excess of the unlabeled 40-bp fragment (a specific competitor) or 100-fold molar excess of the −1166 to −1126 fragment from the *Acs2* promoter (a nonspecific competitor; Figure 2).

To isolate cDNA clones encoding the DNA binding protein(s) specific for the −715 to −675 region, we screened a tomato

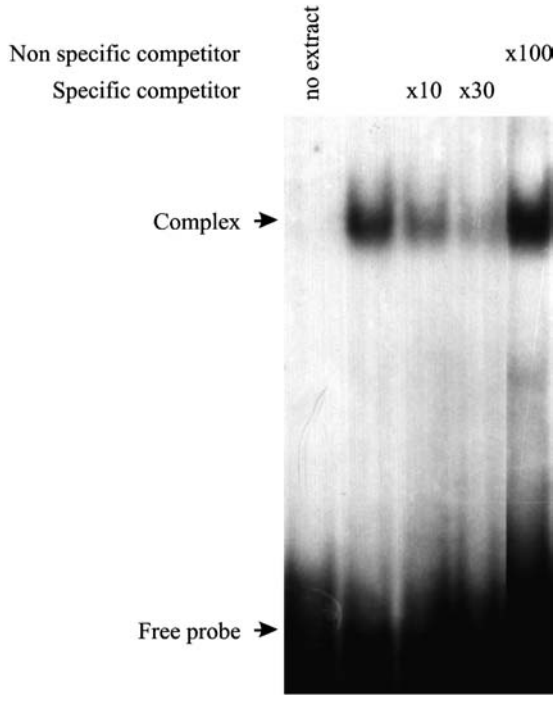

Figure 2. In Vitro Binding of Nuclear Proteins to −715 to −675 of the *Acs2* Promoter.

Gel-shift binding assay with nuclear proteins extracted from EIX-treated tobacco plants that were incubated with 30 fmol of end-labeled 40-bp fragment (corresponding to −715 to −675 in the *Acs2* promoter) in the presence of 2 μg of poly(dI-dC) and 10-, 30-, or 100-fold excess competitor fragments, as indicated. After the binding step, the DNA–protein complexes were fractionated by PAGE and detected by autoradiography. The bound complexes are identified at left.

cDNA expression library using the 40 bp as a probe as previously described (Singh et al., 1988; Katagiri et al., 1989). Screening of 160,000 plaque-forming unit phages yielded the isolation of a single positive phage. The phage contained an insert with an ORF of 481 amino acids. Searching the database with the isolated ORF identified it as a Cys protease–like protein of the clan C13 Cys proteases. The ORF (named LeCp; accession number AJ841791) showed 51 to 87% identity (Figure 3) to Cys proteases from *Nicotiana tabacum* (87%; accession number AB075947), *Citrus sinensis* (73%; accession number P49043), *Arabidopsis thaliana* (73%; accession number Q39119), *Ipomoea batatas* (72%; accession number AAF69014.1), *Canavalia ensiformis* (57%; accession number P49046), and *Homo sapiens* legumain (51%; accession number Q99538).

Gel-shift assays were used to test the interaction between the LeCp protein and the *cis*-acting element described above. The *LeCp* ORF was cloned in frame to the His tag in the pET28 *Escherichia coli* expression vector, and the expressed protein was reacted with the 40-bp fragment (−715 to −675) from the *Acs2* promoter (Figure 4). The specificity of binding was monitored using 2-, 10-, and 25-fold excess of the unlabeled −715 to −675 fragment (specific competitor) or 50- and 250-fold molar excess of the −1166 to −1126 fragment from the *Acs2* promoter (nonspecific competitor). When monoclonal antibodies against the His tag were added to the gel-shift assay mixture, a larger complex of the −715 to −675 LeCp antibodies was observed as a supershift (Figure 4). To further test the specificity of binding between LeCp and the −715 to −675 fragment, we mutagenized the *cis* element. The sequence TAAAATAT in the −715 to −675 region was changed to TCCCATAT. Gel-shift analysis between LeCp and the mutated *cis* element revealed that the mutated *cis* element has significantly reduced binding activity to the LeCp protein compared with the wild-type element (Figure 4).

Our data suggest that LeCp is involved in controlling the expression of *Acs2* by binding to a *cis* element in the −715 to −675 region of the *Acs2* promoter.

In Vivo Activation of *Acs2* by the LeCp

We tested the hypothesis that LeCp functions in controlling *Acs2* expression in vivo. The *LeCp* cDNA was cloned in the sense orientation into the binary vector pBINPLUS (van Engelen et al., 1995) between the 35S-Ω promoter containing the translation enhancer signal and the nopaline synthase (Nos) terminator (Mitsuhara et al., 1996). The construct was electroporated into *Agrobacterium tumefaciens* GV3101 and the bacteria used for transient expression assays. Transient expression of LeCp induced GUS activity in transgenic tomato plants harboring the −3058 promoter upstream of the GUS reporter gene, but transient expression of LeCp failed to induce GUS activity in transgenic plants harboring the −675 or Δ40 deletions upstream of the GUS reporter gene (Figure 5A). Transient expression of the empty vector did not induce GUS activity in transgenic plants harboring the −3058 *Acs2* promoter upstream of the GUS reporter gene (Figure 5A). Transient expression of LeCp induced GUS activity in transgenic tobacco plants harboring the −3058 *Acs2* promoter upstream of the GUS reporter gene, similar to the results obtained in tomato plants (see Supplemental Figure 2

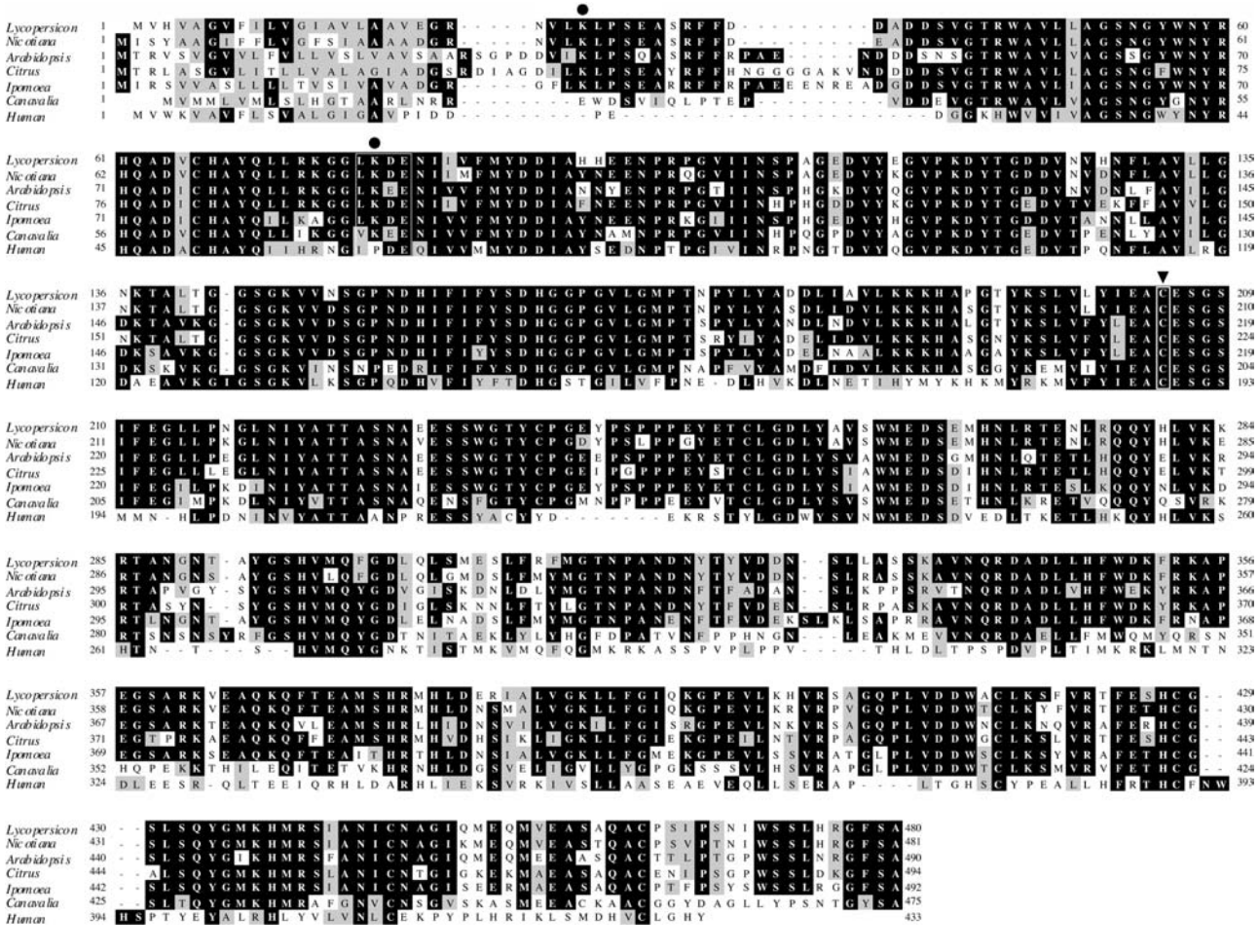

Figure 3. Similarity of LeCp to Cys Proteases.

Alignment of the LeCp amino acid sequence (accession number AJ841791) with those of Cys proteases from *Nicotiana tabacum* (accession number AB075947), *Citrus sinensis* (accession number P49043), *Arabidopsis thaliana* (accession number Q39119), *Ipomoea batatas* (accession number AAF69014.1), *Canavalia ensiformis* (accession number P49046), and *Homo sapiens* legumain (accession number Q99538). The bestfit and pretty box programs (GCG sequence analysis software package, version 10.0; Accelrys, San Diego, CA) were used to create the best alignment. Identical amino acids are shaded in black, and conservative substitutions are shaded in gray. The triangle marks the conserved Cys that is essential for the enzymatic activity. A circle marks the SUMOlation sites.

online). Moreover, we tested the induction of the endogenous *Acs2* by LeCp. Transient expression of LeCp in *L. esculentum* cv VF36 or application of the EIX elicitor induced the expression of *Acs2* (see Supplemental Figure 3 online).

A silencing approach was undertaken to further demonstrate the role of LeCp in controlling the expression of *LeAcs2*. We used a *Tobacco rattle virus* (TRV)-based virus-induced gene silencing (VIGS) approach (Liu et al., 2002a; Burch-Smith et al., 2004) to silence the expression of *LeCp* in tomato plants harboring the −3058 *Acs2* promoter upstream of the GUS reporter gene. A segment of *LeCp* (425 bp from residue 85 to residue 510) was cloned into the pTRV2 vector (Liu et al., 2002a) to generate pTRV2-*LeCp*. A mixture of Agrobacterium cultures containing TRV-RNA1 (pTRV1) and empty pTRV2, pTRV2 carrying tomato phytoene desaturase (*PDS*) (Liu et al., 2002a), or pTRV2-LeCp was infiltrated onto the lower leaves of 3-week-old tomato plants carrying the −3058 *Acs2* promoter upstream of the GUS reporter gene. Four weeks after TRV infection, silenced plants were infiltrated with an Agrobacterium GV3101 strain containing Pro_{35S}:tvEIX. The induction of GUS activity in pTRV2-*LeCp*–silenced tomato plants was significantly lower than the induction of GUS activity in tomato plants silenced with empty vector or pTRV2-*tPDS* (Figure 5B).

LeCp shows high homology with Cys proteases from the C13 clan. It shows 94% similarity to the tobacco VPE having a protease activity (Hatsugai et al., 2004). Sequence alignment of several Cys proteases (Chen et al., 1998) revealed two amino acids that are essential for the catalytic activity of the proteins. Changing one of these amino acids, the Cys-191 residue, to a Ser residue completely inactivated the enzymatic activity of the mammalian legumain (Chen et al., 1998). This protein shows 51% homology with the LeCp protein, and Cys-191 is conserved

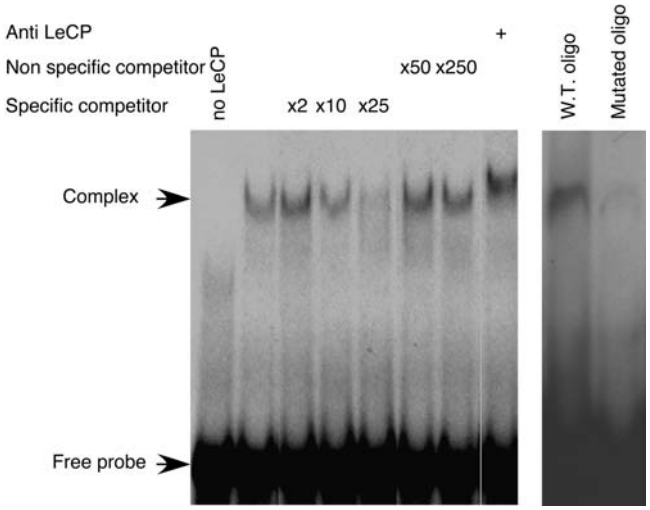

Figure 4. In Vitro Binding of LeCp to Promoter Sequences of *Acs2*.

Gel-shift binding assay was performed by incubating LeCp (0.1 μg) with 30 fmol of end-labeled 40-bp fragment (corresponding to −715 to −675 in the *Acs2* promoter) and 2-, 10-, 25-, 50-, or 250-fold excess competitor fragments, as indicated (left). LeCp (0.1 μg) was also incubated with 30 fmol of end-labeled wild-type (W.T.) or mutated 40-bp fragment (corresponding to −715 to −675 in the *Acs2* promoter), as indicated, in the presence of 2 μg of poly(dI-dC) (right). After the binding step, the DNA–protein complexes were fractionated by PAGE and detected by autoradiography. Bound complexes are identified at left. oligo, oligonucleotide.

in both proteins (Figure 3; Cys-205 in LeCp). Moreover, Cys-191 is conserved in the C13 family of Cys proteases. Therefore, we used site-directed mutagenesis to modify the Cys in the active site. Cys-205 of LeCp was modified to Ser. The mutated *LeCp* was cloned in the binary vector pBINPLUS between the 35S-Ω promoter containing the translation enhancer signal and the Nos terminator. Transient expression of the mutated LeCp protein induced GUS activity in transgenic plants harboring the −3058 promoter but failed to induce GUS activity in transgenic plants harboring the Δ40 deletion (Figure 5C). These experiments indicate that the catalytic activity of LeCp is not required for the induction of *Acs2*. Moreover, our data clearly illustrate that LeCp can induce the expression of *Acs2* only if the −715 to −675 sequence is present in the *Acs2* promoter.

In Vivo Binding of LeCp to the *Acs* Promoter

Tomato and tobacco plants behave similarly in their response to EIX treatment. EIX induces the induction of *Acs* expression in tobacco leaves (Avni et al., 1994). Application of the EIX elicitor to *N. tabacum* cv SR1 (EIX nonresponding plants) expressing the tomato *LeEix2* gene induces hypersensitive response development (Ron and Avni, 2004).

We examined the interaction between LeCp and the *Acs* promoter in vivo. Chromatin immunoprecipitation (ChIP) was used to examine the binding of LeCp to the tobacco *Acs* promoter. The ChIP technique allows the purification of an in vivo–formed complex of DNA binding protein and associated DNA and is one of the best approaches available to confirm direct targets of a DNA binding protein (Weinmann et al., 2002).

Leaves from transgenic tobacco plants overexpressing the LeCp–green fluorescent protein (GFP) fusion protein were ground and fixed with formaldehyde. Cross-linked chromatin was than isolated from sonicated nuclei. Preadsorption with preimmune serum and protein A–Sepharose beads was performed to remove any nonspecific DNA–protein complexes. An aliquot of the solubilized chromatin after preadsorption was used to recover total DNA (input DNA).

Antibodies against GFP were used to immunoprecipitate LeCp-GFP–DNA complexes. Coimmunoprecipitated DNA was extracted and subjected to PCR using primers corresponding

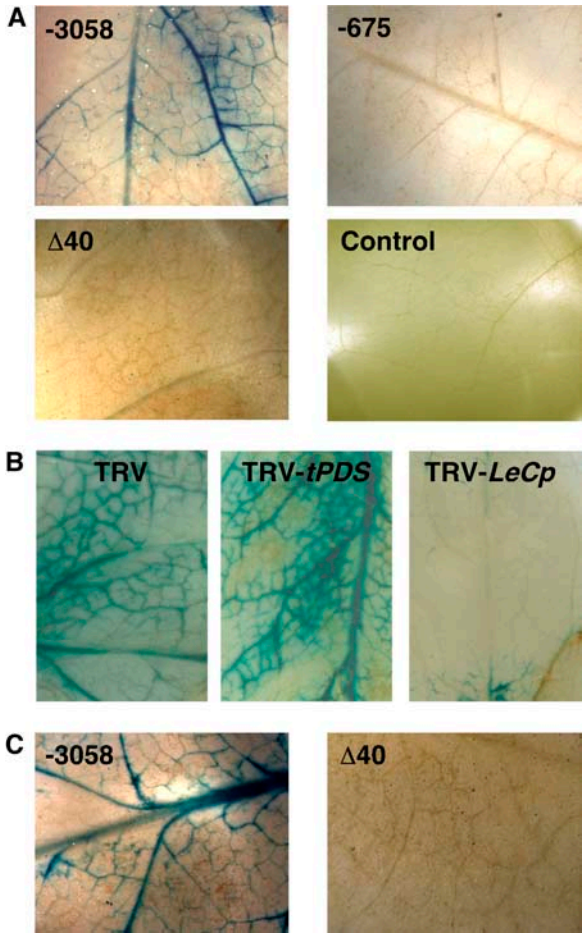

Figure 5. In Vivo Induction of the GUS Reporter Gene by *LeCp* Expression.

Transgenic *L. esculentum* cv VF36 plants harboring the 3058-bp promoter and the deleted promoter fragments (as indicated) cloned upstream of the *uidA* gene were infiltrated with Agrobacterium GV3101 (OD_{600} = 0.1) containing either the Pro_{35S}:LeCp construct or empty vector (control) **(A)**, infected with TRV, TRV-*tPDS*, or TRV-*LeCp* followed by infection with Agrobacterium GV3101 (OD_{600} = 0.1) containing the Pro_{35S}:tvEix construct **(B)**, or infiltrated with Agrobacterium GV3101 (OD_{600} = 0.1) containing the mutated Pro_{35S}:LeCp construct **(C)**. GUS activity was monitored at 48 h after injection.

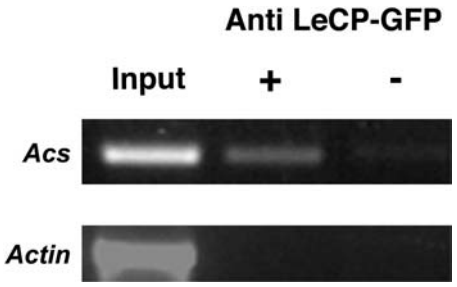

Figure 6. In Vivo Binding of LeCp Protein to Promoter Sequences of the *Acs* Gene.

Transgenic tobacco plants harboring Pro_{35S}:LeCp-GFP were subjected to ChIP with anti-GFP antibodies. PCR was performed using primers corresponding to sequences located near the 40-bp motif in the *Acs2* promoter and primers corresponding to the actin gene. As a control for DNA input into the ChIP assays, aliquots of sonicated chromatin were analyzed by PCR using the same primers as those used in the ChIP assays.

to sequences in the *Acs2* promoter and specific primers for the tobacco actin gene (Figure 6). ChIP assays using anti-GFP antibodies showed that LeCp bound the tobacco *Acs* promoter in vivo. PCR products were generated with *Acs* promoter–specific primers but not with actin-specific primers when DNA–protein complexes (''chromatin'') were immunoprecipitated with anti-GFP antibodies (Figure 6). Specific amplification of *Acs* promoter sequences or the actin sequence could not be detected when nonspecific antibodies were used to immunoprecipitate protein–DNA complexes. Control experiments showed that actin sequences were not present in the immunoprecipitation complex, whereas actin sequences were present in the input DNA as the *Acs* promoter sequences (Figure 6). Moreover, *Acs* promoter sequences were not present in the immunoprecipitation complex when plants overexpressing free GFP were used for the ChIP analysis (data not shown), indicating that LeCp and not GFP binds to the *Acs* promoter. The amplified DNA fragment that coimmunoprecipitated with LeCp was cloned and sequenced. The sequence showed high homology with the *Acs2* promoter in the vicinity of the 40-bp *cis* element (see Supplemental Figure 4 online). The *Acs* promoter–specific primers amplified only a single fragment when tobacco genomic DNA was used as a template. Sequencing the tobacco fragment revealed that it is identical to the DNA fragment that coimmunoprecipitated with LeCp (data not shown). DNA gel blot analysis (data not shown) revealed that the tomato and tobacco *Acs* promoters differ upstream of the *cis* element. Moreover, the *Acs2* promoter–specific primers could amplify only a single fragment when tobacco genomic DNA was used as a template. As the amplified sequence in tobacco is identical to the *Acs2* promoter region, the ChIP experiments in this case may be performed in tobacco.

Interaction between LeCp and the Small Ubiquitin-Related Modifier Protein

SUMO (for small ubiquitin-related modifier) is the best-characterized member of a growing family of ubiquitin-related proteins. It resembles ubiquitin in its structure and its ability to be ligated to other proteins as well as in the mechanism of ligation. However, in contrast with ubiquitination, often the first step on a one-way road to protein degradation, SUMOlation does not seem to mark proteins for degradation (Muller et al., 2001). SUMOlation may alter protein stability or function by competing with ubiquitin for target Lys residues (Hoege et al., 2002). Alternatively, SUMO modification may alter protein–protein interactions, which could have a variety of consequences, including changes in cellular localization (Seeler and Dejean, 2001). In this respect, SUMO modification was shown to regulate the function of the sp3 transcription factor by regulating its cellular localization (Ross et al., 2002).

Bioinformatics analysis indicates that LeCp does not contain a consensus nuclear localization signal. Localization of the LeCp-GFP fusion protein showed that it is found mainly in the cytoplasm (data not shown). This is in agreement with the major activity of the protein being a Cys protease. Therefore, we postulated that SUMO modification of LeCp might regulate its localization. SUMO modification occurs at a particular sequence, ΨKxE (where Ψ represents L, I, V, or F) (Hay, 2001). We used the program SUMOplot (www.abgent.com/doc/sumoplot) to predict SUMOlation sites in LeCp. Two high-score SUMOlation sites were found. The first site is located in the signal peptide, and the second (with the highest score) is located between amino acids 77 and 80 of LeCp (Figure 3). We used the yeast two-hybrid system to test a possible interaction between LeCp and tomato SUMO (T-SUMO). The LeCp protein was sufficient to activate the *lacZ* gene in the yeast two-hybrid system without interaction with any other protein (data not shown). Therefore, we cloned LeCp in the prey plasmid (pJG4-5). T-SUMO and Bicoid (a transcriptionally inert fragment of the Drosophila Bicoid product) were fused in frame to the C terminus of the DNA binding domain of LexA in the ''bait'' plasmid pEG202 and transformed into yeast strain EGY48 containing LeCp (Gyuris et al., 1993) to check for possible interaction with LeCp. Moreover, the ribulose-1,5-bis-phosphate carboxylase/oxygenase (Rubisco) large subunit gene was cloned in the prey plasmid (pJG4-5) to check for possible interaction with T-SUMO.

Yeast strains carrying the T-SUMO bait and LeCp prey grew in the absence of Leu, indicating *LEU2* reporter activation. When grown on X-Gal plates, these yeast cells were blue as a result of *LacZ* reporter activation (Figure 7A). By contrast, a control yeast strain expressing the Bicoid or empty vector (pEG202) and the LeCp prey or yeast strain expressing the T-SUMO in the bait and Rubisco in the prey (pJG4-5) did not activate the *LEU2* or *LacZ* reporter (Figure 7A). Expression was dependent on growth on galactose medium, indicating that expression of both LeCp and T-SUMO are required for expression of the reporter genes. Our data indicate that interaction between LeCp and T-SUMO is required for reporter expression. To test this notion, we changed LKDE (amino acids 77 to 80) to LEDG, disrupting the consensus SUMOlation sequence in LeCp, and challenged the interaction of the mutated protein with T-SUMO in the yeast two-hybrid system. As seen in Figure 7A, wild-type LeCp interacts with T-SUMO but mutated LeCp does not. This finding suggests a direct interaction between the T-SUMO and the LeCp proteins.

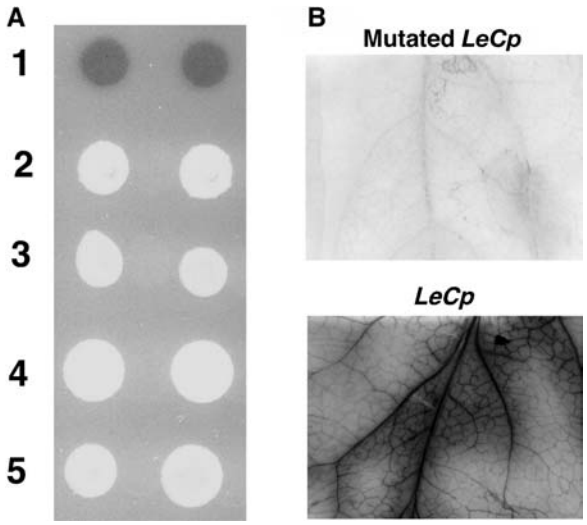

Figure 7. Interaction of LeCp with T-SUMO.

(A) EGY48 yeast cells containing T-SUMO (in pEG202) and LeCp (in pJG4-5) (row 1), T-SUMO (in pEG202) and mutated LeCp (in pJG4-5) (row 2), Bicoid (in pEG202) and LeCp (in pJG4-5) (row 3), empty vector (in pEG202) and LeCp (in pJG4-5) (row 4), or T-SUMO (in pEG202) and Rubisco (in pJG4-5) (row 5) were grown on galactose medium lacking the amino acids His, Ura, and Trp and supplied with X-Gal.

(B) Transgenic *L. esculentum* cv VF36 plants harboring the 3058-bp promoter cloned upstream of the *uidA* gene were infiltrated with Agrobacterium GV3101 (OD_{600} = 0.1) containing either the Pro_{35S}:LeCp construct or the SUMOlation signal–mutated Pro_{35S}:LeCp construct. GUS activity was monitored at 48 h after injection.

We tested whether the SUMOlation signal in LeCp is required for its transcriptional factor activity in vivo. The mutated *LeCp* having a disrupted SUMOlation signal was cloned into the binary vector pBINPLUS between the 35S-Ω promoter containing the translation enhancer signal and the Nos terminator. Transient expression of the wild-type LeCp but not the mutated LeCp protein induced GUS activity in transgenic tomato plants harboring the −3058 promoter upstream of the GUS reporter gene (Figure 7B). The induction of endogenous *Acs2* by the mutated *LeCp* having a disrupted SUMOlation signal was significantly lower compared with that of the wild-type protein (see Supplemental Figure 3 online).

Together, these results show that LeCp can bind in vivo to the *Acs* promoter, suggesting dual functions for this protein: LeCp as a Cys protease and as a gene inducer. We propose that in the plant, LeCp functions as a Cys protease, but upon modification by SUMO, it enters the nucleus and acts as an *Acs* gene inducer.

DISCUSSION

In this study, we identified a novel protein involved in ethylene biosynthesis induction by the fungal elicitor EIX. The enzyme ACC synthase catalyzes the rate-limiting step in ethylene biosynthesis. This enzyme is encoded in tomato by at least eight family members (Alexander and Grierson, 2002). We found that *Acs2* is induced by EIX. These results agree with published data showing that *Acs2* is induced by wounding, ripening, floral senescence, and *Phytophthora infestans* elicitor (Olson et al., 1991; Rottmann et al., 1991; Lincoln et al., 1993; Oetiker et al., 1997).

To identify the *cis* element(s) that regulate *Acs2* expression elicited by EIX, we generated several *Acs2* promoter deletions that were cloned upstream of the GUS reporter gene. We found that the sequence between −715 and −675 is essential for the regulation of *Acs2* expression elicited by EIX. To eliminate the possible involvement of additional upstream elements in the regulation of *Acs2* expression, we generated the Δ40 deletion. EIX treatment did not induce GUS activity in transgenic plants having the Δ40 deletion upstream of the GUS reporter, indicating that only the sequence from −715 to −675 is essential for the regulation of *Acs2* expression elicited by EIX. A search of the database with the sequence of the −715 to −675 element revealed that it exists in two different promoters of genes involved in plant defense responses (Reuber and Ausubel, 1996; Pontier et al., 2001). Gel retardation experiments confirmed that nuclear proteins bind to the −675 to −715 region, making it a candidate to isolate *trans*-acting proteins that regulate the EIX-mediated expression of *LeAcs2*.

Using the *cis* element, we isolated a cDNA representing a Cys protease protein (LeCp) that binds to the AT-rich element in the *Acs2* promoter. Gel retardation assays confirmed that the LeCp protein binds to the 40-bp fragment. Moreover, altering the sequence in the core element to TCCCAT had a negative effect on LeCp protein binding, indicating specific in vitro binding of LeCp to the −715 to −675 region in the *Acs2* promoter.

To demonstrate the in vivo role of LeCp in regulating the *Acs2* gene, we transiently expressed the *LeCp* gene in transgenic plants harboring the *Acs2* promoter upstream of the GUS reporter. As in the case of the promoter deletion analysis, the induction of GUS reporter expression suggested specific regulation of *Acs2* by LeCp. Moreover, mutating the putative catalytic active site of LeCp did not affect its ability to specifically induce the expression of the reporter gene, thus suggesting that the LeCp putative protease active site is not required for the interaction between *Acs2* and LeCp. The in vivo presence of LeCp inside the nuclei was shown by ChIP analysis.

Cys proteases have been shown to be involved in different aspects of plant defense (Estelle, 2001; Staskawicz et al., 2001; Elbaz et al., 2002; Hotson et al., 2003; Hatsugai et al., 2004). The LeCp protein shows homology with the legumain Cys endopeptidases, which show similar protein folding to animal caspases that mediate programmed cell death in mammals (Chen et al., 1998). LeCp shows 94% similarity to *N. tabacum* VPE, which exhibits caspase-1 activity. The protease is essential for a virus-induced hypersensitive response and is involved in programmed cell death (Hatsugai et al., 2004). The tomato *Rcr3*, which encodes a Cys protease, was shown to be a positive regulator of *Cf-2*–dependent resistance (Kruger et al., 2002). Moreover, Cys proteases have been shown to be induced during different plant stresses (Alonso and Granell, 1995; Kinoshita et al., 1999; Harrak et al., 2001; Pontier et al., 2001).

To test the in vivo interaction between LeCp and the *Acs* promoter, we performed ChIP analysis. Our data clearly demonstrate the in vivo interaction between the LeCp protein and

sequences in the *Acs* promoter, suggesting that LeCp acts as a dual-function protein.

An increasing number of proteins have been found to have more than one function and are referred to as "moonlighting" proteins (Jeffery, 1999, 2003; Ejiri, 2002). Many enzymes have been found to moonlight (Jeffery, 1999), having additional functions that generally are not enzymatic but rather structural or regulatory. For example, the PutA flavoprotein from *E. coli* serves as both a Pro dehydrogenase and a transcriptional regulator (Vinod et al., 2002). The transcription factor Eyes absent has intrinsic protein Tyr phosphatase activity in addition to its function as a transcription factor (Tootle et al., 2003). Moreover, metabolic enzymes were shown to directly bind and regulate eukaryotic gene expression (Hall et al., 2004). Here, we show an additional function for a Cys protease, LeCp, suggesting that it is a moonlighting protein. LeCp may act as a Cys protease in the cytoplasm and have a role in the hypersensitive response and vacuolar processing and may also possess a second function as a protein that binds to the *Acs2* promoter and induces its expression.

LeCp does not contain a nuclear localization signal. We propose that SUMO modification of LeCp generates the signal required for LeCp to enter the nucleus. SUMO covalently attaches to Lys residues in the target proteins, thereby changing the properties of the modified protein (Girdwood et al., 2004; Salinas et al., 2004; Terui et al., 2004). At present, 60 different mammalian proteins have been shown to be targets for SUMOlation. More than half of the identified SUMO substrates are known to act as coactivators or corepressors of transcription or are transcription factors themselves (Girdwood et al., 2004). Our data indicate that SUMO may modify LeCp. This modification changes the activity of the protein, enabling it to enter the nucleus and exhibit its transcriptional factor activity, which induces the *Acs2* gene.

It should be noted that we are dealing with a minor function of the LeCp protein, the major activity being its action as a protease (VPE). Therefore, we anticipate that only small amounts of protein are modified by SUMO in vivo. Moreover, there are many stable SUMO-deconjugating enzymes, making modification of LeCp by SUMO difficult to discern in vivo. However, we believe that the yeast two-hybrid experiment and the mutation experiments clearly demonstrate an interaction between the two proteins.

We suggest that upon application of the EIX elicitor, SUMO modifies LeCp, enabling it to enter the nucleus and activate the expression of *Acs*.

METHODS

Plasmid Constructs and Plant Transformation

The plasmid (pCB1-155) containing the *uidA* (GUS) gene under the control of the tomato (*Lycopersicon esculentum*) *Acs2* 3058-bp promoter was kindly given by A. Theologis (USDA Plant Gene Expression Center, Albany, CA) (Rottmann et al., 1991). For promoter deletion analysis, pCB1-55 was cut with *Hin*dIII and *Sca*I. The fragment containing the *LeAcs2* and *uidA* genes was cloned into pBluescript SK+ vector (Stratagene, La Jolla, CA). Deletion clones −330, −807, and −675 were created using the Exo-size kit (New England Biolabs, Beverly, MA) according to the manufacturer's protocol. Deletion clones −770 and −715 were prepared by PCR using specific primers. For deletion −770, we used primers 5′-CCCAAGCTTGGTTTTGACAGG-3′ and 5′-GGGGATCCATTTTTTTTTTACTAAATG-3′. For deletion −715, we used primers 5′-CCCAAGCTTGGTAAAATATATTTTTTTG-3′ and 5′-GGGGATCCATTTTTTTTTACTAAATG-3′. The full-length promoter and the deleted promoters were cloned into the binary vector pBINPLUS (van Engelen et al., 1995) containing the *uidA* gene.

Four different primers were used to create Δ40. Primers 5′-CGGGATCCTCATTACTTGTCTATG-3′ and 5′-CAAAAAAATATATTTTACTGCAGTATATCA-3′ were used to amplify the −715 to −3058 fragment of the *Acs2* promoter. Primers 5′-GGCTGCAGGGGTAAAAATAATTAAAAC-3′ (containing the *Pst*I site) and 5′-GGGGATCCATTTTTTTTTTACTAAATG-3′ (containing the *Bam*HI site) were used to amplify the 0 to −675 fragment of the *LeAcs2* promoter. The PCR fragments were cut with *Bam*HI and *Pst*I and cloned into the binary vector pBINPLUS (van Engelen et al., 1995) containing the *uidA* gene.

L. esculentum cv VF36 plants were transformed as described previously (McCormick, 1991).

EIX Treatments

Xylanase (Fluka, Milwaukee, WI) was purified as described previously (Dean and Anderson, 1991). EIX (2 to 5 μg/mL) was injected into leaves using needleless syringes as described previously (Hanania et al., 1999). Alternatively, EIX was applied as a hanging drop to freshly cut petioles of detached leaves (2.5 μg/g tissue) as described (Avni et al., 1994). The induction of GUS activity was monitored at 4 h after treatment.

Fluorimetric and Histochemistry Assays

Excised leaf tissues were assayed for GUS activity as described previously (Jefferson et al., 1987). Proteins were extracted and incubated in the presence of 1 mM 4-methylumbelliferyl β-D-glucuronide at 37°C. Samples were taken at time 0, and after 1 h, the enzymatic reaction was stopped with 0.2 M Na_2CO_3. The fluorometer (FL500) was calibrated with 10, 20, 50, and 100 nM 4-methylumbelliferyl P-D-glucuronide. Protein concentration was determined according to Bradford (1976).

For histochemical detection, tissues from transgenic plants were incubated overnight at 37°C in 1 mM 5-bromo-4-chloro-3-indolyl 6-β-glucuronide, 25 mM sodium phosphate buffer, pH 7.0, and 1% DMSO. To stop the reaction and to remove the chlorophyll, the tissues were treated with ethanol for 24 h.

Gel Retardation Assays

Nuclear proteins from tobacco (*Nicotiana tabacum*) leaves were extracted as described previously (Meller et al., 1993). The 40-bp primer (−715 to −675) from the *LeAcs2* promoter was end-labeled using T4 polynucleotide kinase (Sambrook et al., 1989). Two complementary primers were annealed to generate a double-stranded DNA probe for the binding reaction. Binding reactions (30 μL) contained 30 fmol of the end-labeled DNA probe, 4 mM Hepes, pH 7.9, 3 mM $MgCl_2$, 1 mM DTT, 10% glycerol, 1 μg of BSA, 8 mM KCl, 0.02 mM EDTA, 2 μg of double-stranded poly (dI-dC), and competitor DNA sequences, as indicated. Reactions were initiated by the addition of total nuclear extract (0.7 μg/μL) or purified LeCp protein (1 μg). After 20 min of incubation at room temperature, DNA binding activities were resolved on 7% polyacrylamide gels in 0.5× Tris borate–EDTA buffer. Gels were dried on Whatman paper and autoradiographed.

Screening of an Expression Library for Proteins Interacting with the *Acs2* Promoter

Forward and reverse primers of the −715 to −675 sequence of the *Acs2* promoter were mixed in the same molar ratios to form the double-stranded element. The double-stranded element was self-ligated to produce a multiple 40 repetitive element (average of eight repetitions). The poly-40 element was labeled using the specific primers 5′-CTAAAA-TATATTTTTTTG-3′ and 5′-CCAACCAACCCCCAACAACCAAA-3′.

An *L. esculentum* mixed elicitor library obtained from Greg Martin was screened with the poly-40 element. Phages (160,000 plaque-forming units) were grown for 3 h at 37°C. A nitrocellulose filter containing 1 mM isopropylthio-β-galactoside was subsequently placed on each plate, and the phages were grown for an additional 4 h.

The nitrocellulose filters were lifted from the plates and blocked for 2 h at 4°C with 50 mM Tris-Cl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, and 5% low-fat milk, followed by a short wash with 10 mM Tris-Cl, pH 7.5, 50 mM NaCl, 1 mM EDTA, and 1 mM DTT (binding buffer). Filters were hybridized overnight at room temperature with 2 ng/mL labeled poly-40 DNA (1 to 2 cpm/mL). Filters were then washed with a large excess of binding buffer for 1 h at room temperature and exposed to film.

Sequence Analysis

DNA sequencing was performed using the dideoxy chain-termination method (Sanger, 1981). Sequence analysis was performed using the GCG sequence analysis software package (version 10.0; Accelrys). The BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST/) was used to search the DNA and protein databases for similarity. Motifs were identified using the SMART (http://smart.embl-heidelberg.de/), the PROSITE (http://www.expasy.ch/prosite), and the SUMOplot (www.abgent.com/doc/sumoplot) programs.

Expression of *LeCp* in *Escherichia coli* Cells

The *LeCp* gene was amplified using primers 5′-GCAGCATATGGTT-CACGTCGCC-3′ and 5′-CCGCAGGATCCATGGCGTTTTACGC-3′ to generate *Nco*I and *Bam*HI restriction sites, respectively. The amplified gene was cloned into the pET28a expression vector (Novagen, Madison, WI). Protein expression was done in the BL21(DE3)pLysS strain of *E. coli*. Cells were grown at 37°C until OD_{600} reached 0.5. Cultures were then induced with 0.4 mM isopropylthio-β-galactoside and allowed to grow for an additional 3 h at 30°C before harvest. LeCp was purified by binding to Ni^{2+}-charged Sepharose beads (Pharmacia Biotech, Piscataway, NJ). Purified protein was used in the gel retardation experiments.

Transient Expression Assay

For transient expression assays, the *LeCp* constructs cloned in pBINPLUS (van Engelen et al., 1995) were introduced by electroporation into *Agrobacterium tumefaciens* strain GV3101. Agrobacterium was grown in Luria-Bertani medium overnight, diluted into induction medium (50 mM Mes, pH 5.6, 0.5% [w/v] glucose, 1.7 mM NaH_2PO_4, 20 mM NH_4Cl, 1.2 mM $MgSO_4$, 2 mM KCl, 17 μM $FeSO_4$, 70 μM $CaCl_2$, and 200 μM acetosyringone), and grown for an additional 6 h until OD_{600} reached 0.4 to 0.5. The Agrobacterium culture was diluted to $OD_{600} = 0.1$, and the suspensions expressing LeCp were injected with a needleless syringe into the leaves of 7- to 8-week-old tomato plants for GUS activity analysis.

VIGS Assay and Virus Infection

VIGS assays were performed by following the protocol described by Liu et al. (2002b). pTRV1, pTRV2, and pTRV-*tPDS* VIGS vectors (described in Liu et al., 2002a) were obtained from S. Dinesh-Kumar at Yale University (New Haven, CT). A segment of the *LeCp* (425 bp from residue 85 to residue 510) was amplified by PCR using primers 5′-GCTGCTCGA-GATGGTTCACGTCGCCG-3′ and 5′-CCGGCTCGAGCGGGAGCAGTT-TTGTTACCAAGGAG-3′ and cloned into the pTRV2 vector (Liu et al., 2002a) to generate pTRV2-*LeCp*. Twenty-eight days after TRV infection, the upper leaves of the plants were infected with Agrobacterium culture expressing Pro_{35S}:tvEIX (Ron and Avni, 2004).

Chromatin Immunoprecipitation

Leaf tissue (3 g) of LeCp-GFP–expressing *N. tabacum* was ground and fixed with 1% formaldehyde in PBS at room temperature for 10 min with gentle mixing. Homogenates were washed twice with ice-cold PBS, resuspended in 100 mM Tris-HCl, pH 9.4, 10 mM DTT, and incubated for 15 min at 30°C. Extracts were sequentially washed twice with 10 mL of ice-cold PBS, once with 0.25% Triton X-100, 10 mM EDTA, 0.5 mM EGTA, and 10 mM Hepes, pH 6.5, and once with 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, and 10 mM Hepes, pH 6.5. Pellets containing mostly nuclei were then suspended in 2 mL of lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris-HCl, pH 8.0) and sonicated four times for 10 s followed by centrifugation for 10 min at 20,000*g* at 4°C. Supernatants were collected, diluted in buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, and 20 mM Tris-HCl, pH 8.0) and immunocleared with 2 μg of sheared salmon sperm DNA, preimmune serum, and protein A–Sepharose (Pharmacia Biotech) for 2 h at 4°C. Immunoprecipitation was performed overnight at 4°C with an anti-GFP polyclonal antibody (Chemicon, Temecula, CA). Precipitates were sequentially washed for 10 min each in TSE I (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, and 150 mM NaCl), TSE II (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, and 500 mM NaCl), and buffer III (0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, and 10 mM Tris-HCl, pH 8.0) followed by three additional washes in 1% SDS and 0.1 M $NaHCO_3$. Pellets were incubated overnight at 65°C to reverse cross-linking. Precipitated DNA fragments were extracted with phenol and precipitated with ethanol.

PCR analysis was performed using equal DNA amounts with the following primers: for the *Acs* promoter, 5′-GGGGATCCATTTTTTTTT-TACTAAATG-3′ and 5′-GACAATCGAATTAGGATAAAAC-3′; for the actin gene, 5′-GAAATCGTGAGGGATGTGAAGG-3′ and 5′-AGAAGCATTTT-CTGTGCACAATGG-3′. Amplified products were analyzed on 1.5% agarose gels.

Yeast Two-Hybrid Interaction

The plasmids (pEG202 and pJG4-5) and yeast strain EGY48 (ura3, his3, trp1, lexApo-leu2) were kindly provided by R. Brent (Massachusetts General Hospital, Boston, MA). The basic procedure for the two-hybrid system was according to Gyuris et al. (1993). To create the in-frame LexA–T-SUMO fusion construct, we cloned the T-SUMO gene into the pEG202 vector. The bait vector construct was confirmed by DNA sequencing. The *LeCp* and Cys-to-Ser *LeCp* genes were cloned separately in the pJG4-5 vector.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession number AJ841791 (*LeCp*).

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