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Ectopic Expression of an Esterase, Which is a Candidate for the Unidentified Plant Cutinase, Causes Cuticular Defects in *Arabidopsis thaliana*

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Cutinase is an esterase that degrades the polyester cutin, a major component of the plant cuticle. Although cutinase activity has been detected in pollen, the genes encoding this enzyme have not been identified. Here, we report the identification and characterization of *Arabidopsis CDEF1* (cuticle destructing factor 1), a novel candidate gene encoding cutinase. *CDEF1* encodes a member of the GDSL lipase/esterase family of proteins, although fungal and bacterial cutinases belong to the α/β hydrolase superfamily which is different from the GDSL lipase/esterase family. According to the AtGenExpress microarray data, *CDEF1* is predominantly expressed in pollen. The ectopic expression of *CDEF1* driven by the 35S promoter caused fusion of organs, including leaves, stems and flowers, and increased surface permeability. Ultrastructural analysis revealed that the cuticle of the transgenic plants was often disrupted and became discontinuous. Subcellular analysis with green fluorescent protein (GFP)-tagged CDEF1 showed that the protein is secreted to the extracellular space in leaves. The recombinant CDEF1 protein has esterase activity. These results are consistent with cutinase being secreted from cells and directly degrading the polyester in the cuticle. *CDEF1* promoter activity was detected in mature pollen and pollen tubes, suggesting that CDEF1 is involved in the penetration of the stigma by pollen tubes. Additionally, we found *CDEF1* expression at the zone of lateral root emergence, which suggests that CDEF1 degrades cell wall components to facilitate the emergence of the lateral roots. Our findings suggest that *CDEF1* is a candidate gene for the unidentified plant cutinase.

Keywords: *Arabidopsis thaliana* • Cuticle • Cutinase • GDSL lipase/esterase family • Lateral root • Pollen.

Abbreviations: CDEF1, cuticle destructing factor 1; GFP, green fluorescent protein; pNPB, *p*-nitrophenyl butyrate; RT–PCR, reverse transcription–PCR; SP, signal peptide; TB, toluidine blue.

Introduction

The plant cuticle is a hydrophobic layer covering nearly all of the above-ground parts of terrestrial plants. The cuticle is deposited at the outer surface of epidermal cells and separates the plant body tissues from the outer environment. The cuticle functions as a surface barrier to protect plants from excessive water loss, pathogens and organ fusion (Jenks et al. 2002, Nawrath 2002, Nawrath 2006). The outermost surface of the cuticle is covered with epicuticular waxes, while the inner layer of the cuticle consists of cutin matrix and intracuticular waxes (Nawrath 2006). Waxes are composed of mainly aliphatic lipids, such as very long-chain fatty acids and their derivatives (Jetter and Schaffer 2001, Kunst and Samuels 2003, Samuels et al. 2008). Cutin is a polyester composed mainly of C_{16} and C_{18} hydroxy and epoxy fatty acids (Kolattukudy 2001, Heredia 2003, Pollard et al. 2008).

Cutinase is an esterase that degrades cutin. This enzyme was first isolated from the fungus *Fusarium solani* sp. (Soliday and Kolattukudy 1976, Soliday et al. 1984). Recently, a cutinase from the bacterium *Thermobifida fusca* was also identified (Chen et al. 2008). Bacterial and fungal cutinases are thought to function by degrading the plant cuticle during infection of a plant. Indeed, cutinases are necessary for some fungi to exhibit full virulence (Schafer 1993, Rogers et al. 1994, Kolattukudy et al. 1995, Li et al. 2003, Skamnioti and Gurr 2007) and the *Magnaporthe grisea* Cutinase2 protein has been demonstrated to be involved in host penetration (Skamnioti and Gurr 2007). The molecular masses of most identified cutinases of fungi and bacteria are approximately 20 kDa (Longhi and Cambillau 1999, Egmond and de Vlieg 2000, Chen et al. 2008). These cutinases have a nucleophilic serine in the active site, which consists of the consensus sequence Gly-X-Ser-X-Gly in the primary structure, similar to the classical esterases and lipases (Carvalho et al. 1999). They also degrade synthetic esters and lipase substrates in vitro (Carvalho et al. 1999). All of the cutinases identified

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thus far belong to the α/β hydrolase superfamily (Carvalho et al. 1999, Longhi and Cambillau 1999, Chen et al. 2008), which consists of various types of enzymes, such as proteases, esterases and transferases (Holmquist 2000), and which have a broad range of substrate specificities, including esters, peroxides, epoxides and even C–C bonds (Holmquist 2000).

No cutinase genes have yet been identified in plants. However, extracts from plants are known to be capable of degrading the cuticle (Shayk and Kolattukudy 1977, Maiti et al. 1979, Hiscock et al. 1994). In plants of dry stigma species, the cuticle on the surface of the stigma is destroyed after pollination, to facilitate penetration of the sigma by pollen tubes (Dickinson and Lewis 1973, Heslop-Harrison and Heslop-Harrison 1975, Knox et al. 1976, Heslop-Harrison 1977, Heslop-Harrison and Heslop-Harrison 1981, Elleman et al. 1992, Hiscock et al. 2002). Many species, including cruciferous plant species, achieve this process by enzymatic degradation of the stigma cuticle (Dickinson and Lewis 1973, Heslop-Harrison and Heslop-Harrison 1975, Knox et al. 1976, Heslop-Harrison 1977, Heslop-Harrison and Heslop-Harrison 1981). These studies suggest that an enzyme capable of degrading the cuticle, such as cutinase, is involved in the pollen–stigma interaction. Indeed, two proteins exhibiting cutinase activity have been isolated from pollen (Shayk and Kolattukudy 1977, Maiti et al. 1979, Hiscock et al. 1994). The first one, from *Tropaeolum majus*, has a molecular mass of 40 kDa (Maiti et al. 1979), which is much higher than the molecular masses of previously identified fungal and bacterial cutinases. Additionally, this pollen cutinase was completely resistant to the serine type of esterase inhibitors, but was efficiently inactivated by thiol-directed inhibitors (Shayk and Kolattukudy 1977, Maiti et al. 1979), suggesting that this enzyme is a cysteine type esterase and is completely different from those isolated from fungi and bacteria. The amino acid sequence of this enzyme has not been determined. The second protein, isolated from *Brassica napus*, has a molecular mass of 22 kDa (Hiscock et al. 1994), which was identical to that of the *Fusarium* cutinase, and was recognized by an anti-*Fusarium* cutinase antibody (Hiscock et al. 1994). The amino acid sequence of this enzyme has also not been determined, and no cutinase homologs have been found in other plants. The pollen cutinases from *T. majus* and *B. napus* are active before pollination (Shayk and Kolattukudy 1977, Maiti et al. 1979, Hiscock et al. 1994). In addition, several studies imply the existence of another type of cutinase that is activated by pollination (Heslop-Harrison and Heslop-Harrison 1975, Heslop-Harrison 1977). Although there seem to be at least three types of pollen cutinases, the molecular nature of pollen cutinases remains to be elucidated. Identification of the genes encoding plant cutinases is important for elucidating the mechanism of cuticular digestion by the plants themselves, such as the digestion of the cuticle during the pollen–stigma interaction.

Here, we report the isolation and characterization of *CDEF1* (*CUTICLE DESTRUCTING FACTOR* 1), a candidate gene encoding a cutinase that belongs to the GDSL lipase/esterase family. We found that ectopic expression of *CDEF1* causes cuticular defects in *Arabidopsis thaliana*. We also analyzed the subcellular localization, enzyme activity and promoter activity of CDEF1. Our findings suggest that CDEF1 is a plant cutinase expressed specifically in pollen.

Results

In silico screening for candidate genes of Arabidopsis cutinases

Expression of a fungal cutinase in *A. thaliana* disrupted the cuticle of the plant, leading to the fusion of various organs. (Sieber et al. 2000). This observation suggests that the expression of cutinase in aerial parts of a plant may abolish normal cuticular development. Additionally, endogenous cutinase activity in plants has been reported only in pollen (Shayk and Kolattukudy 1977, Maiti et al. 1979, Hiscock et al. 1994). These results imply that plant cutinase genes are expressed specifically in restricted plant tissues, such as pollen. To identify plant cutinases, we examined two gene families, the α/β hydrolase superfamily and the GDSL lipase/esterase family. Arabidopsis contains 327 genes of the α/β hydrolase superfamily and 111 genes of the GDSL lipase/esterase family. We constructed a heat map of gene expression profiles of the α/β hydrolase superfamily using developmental data from AtGenExpress (Schmid et al. 2005) (**Supplementary Fig. S1**) and selected four genes (*At2g05850*, *At3g52000*, *At3g54240* and *At3g55180*) that are specifically expressed in mature pollen. We also generated a heat map of gene expression profiles of the GDSL lipase/esterase family (**Supplementary Fig. S2**) and identified two genes (*At2g19050* and *At4g30140*) that are specifically expressed in mature pollen.

Ectopic expression of At4g30140 causes defects in cuticular function

We hypothesized that the ectopic expression of a plant cutinase would cause cuticular defects, similar to those observed upon expression of the *Fusarium* cutinase (Sieber et al. 2000). We cloned a cDNA for each of the candidate genes from Arabidopsis flowers, ectopically expressed the proteins in vegetative organs, and established multiple lines for each transgenic plant.

We examined cuticular function using a toluidine blue (TB) test. Plants with an abnormal cuticle are readily stained with TB, because of the increased permeability of their surface (Tanaka et al. 2004). Transgenic plants expressing *At2g05850*, *At2g19050*, *At3g52000*, *At3g54240* or *At3g55180* were not stained with TB. In contrast, transgenic plants with *35S::At4g30140* were stained strongly with TB (**Fig. 1A**, right): we established seven independent lines carrying *35S::At4g30140*, and all these transgenic plants were stained with TB (**Supplementary Fig. S3**). This result suggests that these transgenic plants have a defect in the cuticle. We named *At4g30140 CUTICLE DESTRUCTING FACTOR 1* (*CDEF1*) and further characterized the protein.

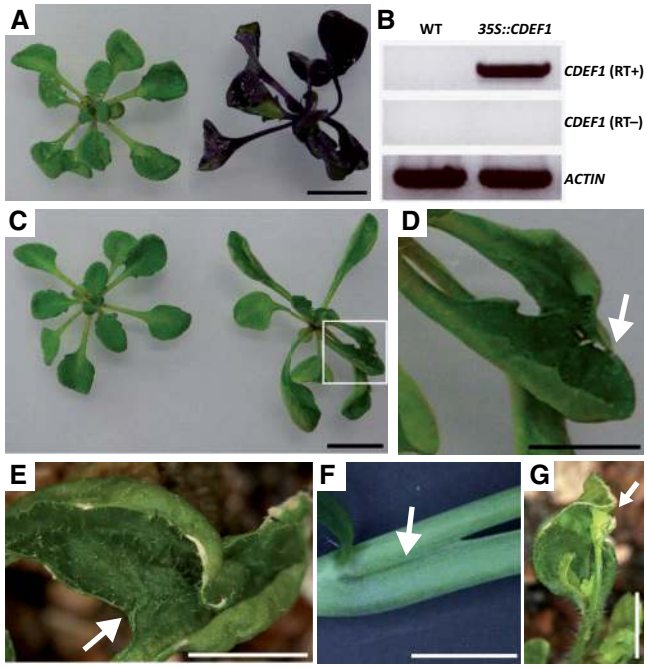

Fig. 1 Ectopic expression of *CDEF1* (*At4g30140*) causes loss of cuticular function. (A) Twenty-one-day-old wild-type and transgenic *35S::CDEF1* plants were subjected to the TB test. Transgenic plants (right) stained strongly with TB, while wild-type plants (left) did not. (B) RT–PCR analysis showing the expression of *CDEF1* mRNA in the leaves of transgenic *35S::CDEF1* plants. Total RNA from true leaves of 14-day-old wild-type (WT) and the transgenic plants was used. *ACTIN* was used as a control. (C) Transgenic *35S::CDEF1* plants (right) occasionally showed an organ fusion phenotype. The fused leaves are enclosed by a white square. Such a phenotype was not observed in wild-type plants (left). (D) Magnified view of the white square in (C). (E–G) Organ fusion of transgenic *35S::CDEF1* plants occurred between the following organs: leaf to leaf (E); stem to stem (F); and leaf to flowers (G). White arrows indicate the boundary between fused organs. Bars = 1 cm (A and C) and 0.5 cm (D–G).

To confirm the expression of the *CDEF1* gene in leaves of the transgenic *35S::CDEF1* plant, we performed a reverse transcription–PCR (RT–PCR) analysis. The *CDEF1* gene was expressed in leaves of transgenic *35S::CDEF1* plants, but not in those of wild-type plants (**Fig. 1B**). We found that transgenic *35S::CDEF1* plants occasionally exhibited organ fusions (**Fig. 1C, D**), while wild-type plants never had fused organs (**Fig. 1C**). This phenotype is strongly correlated with the structural abnormalities of the cuticle (Sieber et al. 2000, Chen et al. 2003, Kurdyukov et al. 2006). The organ fusions occurred in various organs, including leaves (**Fig. 1E**), stems (**Fig. 1F**) and flowers (**Fig. 1G**), suggesting that the cuticular structure was disordered in the organs of the transgenic *35S::CDEF1* plants. Fusions of the organs of flowers prevented normal flower development and such a fused flower did not normally develop a silique, resulting in the reduction of fertility. A similar phenotype was observed in the case of ectopic expression of *Fusarium* cutinase (Sieber et al. 2000). Overall, these results suggest that the ectopic expression of *CDEF1* causes the loss of cuticular integrity in vegetative organs, such as leaves, stems and flowers.

Deformed cuticle in transgenic plants expressing CDEF1

To examine the integrity of the cuticle, leaf epidermal cells of transgenic plants were examined by electron microscopy. In this analysis, we used transgenic Arabidopsis plants expressing a green fluorescent protein (GFP)–CDEF1 fusion protein that caused cuticular defects similar to those seen in plants constitutively overexpressing *CDEF1* (see **Fig. 3B, C**). The cuticle of wild-type plants appeared to be a smooth and continuous layer with a high electron density (**Fig. 2A, B**). In contrast, the cuticle of transgenic plants was often disrupted and became discontinuous. The appearance of the deformed cuticle varied among the specimens examined. The cuticle often disappeared (**Fig. 2C, D**). In some areas, the cuticle was torn (**Fig. 2C, E–G**) and cell wall materials were deposited between the torn cuticular layers (**Fig. 2F, G**). These observations suggest that ectopic expression of the *CDEF1* gene causes disruption of the cuticle.

CDEF1 is secreted into the extracellular space

A hydropathy plot analysis revealed the existence of a possible signal peptide (SP), which could sort this protein into the secretory pathway, at the N-terminus of the CDEF1 protein (**Fig. 3A**). To investigate the subcellular localization of CDEF1 by monitoring GFP fluorescence, the *SP-GFP-CDEF1* fusion gene was expressed in Arabidopsis. The transgenic plants stained readily with TB and occasionally exhibited an organ fusion phenotype (**Fig. 3B, C**), indicating that GFP did not affect the localization or activity of CDEF1. GFP signals in the leaves of 14-day-old transgenic plants were detected in the extracellular space (**Fig. 3D, E**). This result indicates that CDEF1 is secreted and accumulates in the extracellular space.

Recombinant CDEF1 protein has esterase activity

Cutinases are esterases that cleave various mono- and polyesters, including synthetic esters, and native cutin. **Fig. 4** shows an alignment of the amino acid sequences of CDEF1 and GLIP1, a GDSL lipase/esterase protein from Arabidopsis, which was previously reported to exhibit esterase activity (Oh et al. 2005). This alignment revealed that CDEF1 has conserved motifs typical of the GDSL lipase/esterase family of proteins and contains the invariant residues that form the catalytic triad and oxyanion hole (**Fig. 4**). This result implies that CDEF1 has esterase activity.

To verify the enzymatic activity of the protein, we expressed Arabidopsis CDEF1 in suspension cultures of BY-2 tobacco cells and examined the enzymatic activity. The recombinant CDEF1 protein with a 6×His tag at its C-terminus was efficiently expressed in BY-2 cells and secreted into the culture medium. Recombinant CDEF1 was purified with an Ni^{2+} affinity column and appeared as a single band on a Coomassie-stained SDS–polyacrylamide gel (**Fig. 5A**). Purified CDEF1 exhibited esterase activity towards *p*-nitrophenyl butyrate (pNPB) (**Fig. 5B**) and

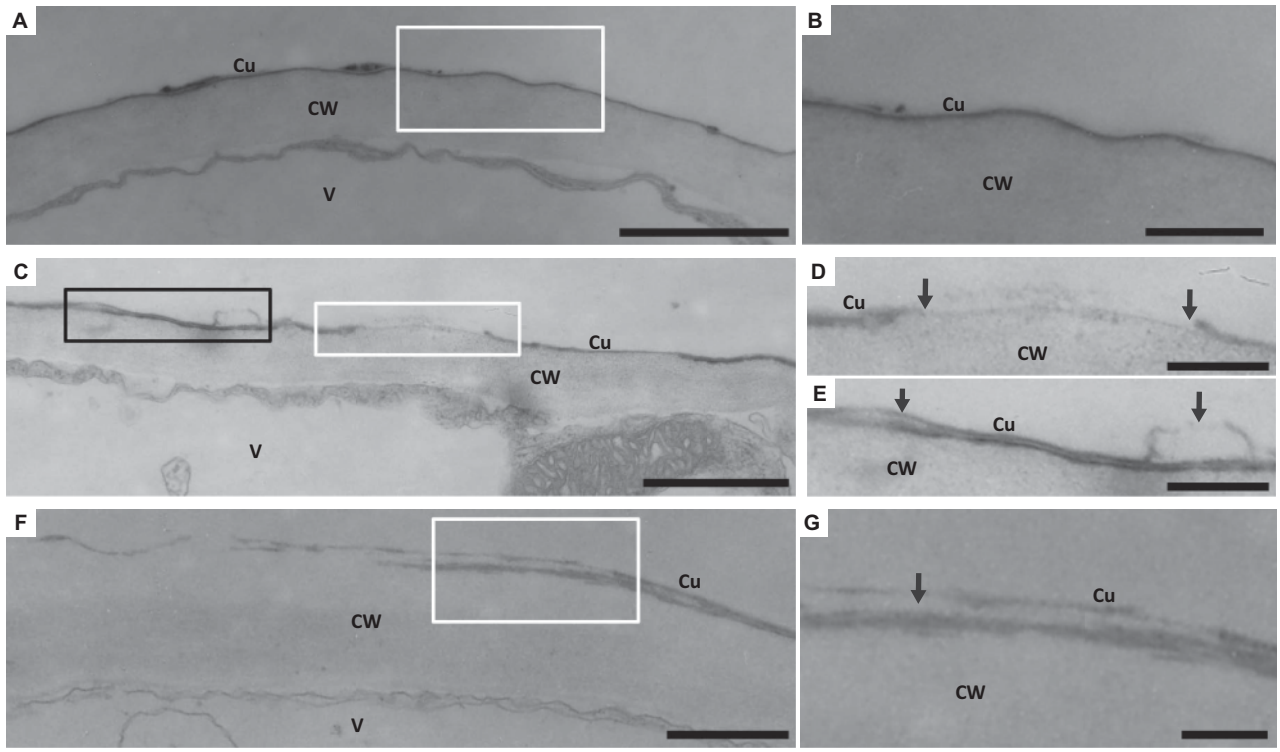

Fig. 2 The cuticle of transgenic plants expressing *CDEF1* is disrupted. (A–G) Electron micrographs of the leaf cuticle of wild-type (A and B) and transgenic *35S::SP-GFP-CDEF1* plants (C–G). (A) The cuticle of wild-type plants forms a thin and smooth layer that has a high electron density. (B) Magnified view of the white square in (A). (C) The cuticle of the transgenic plants was often absent in some regions (see the region enclosed by the white square). In some regions, the cuticle was torn (black square). (D) Magnified view of the region enclosed by the white square in (C). The cuticle is absent in the region between the black arrows. (E) Magnified view of the region enclosed by the black square in (C). The black arrows indicate the torn cuticle. (F) Severely torn cuticle of transgenic plants. (G) Magnified view of the region enclosed by the white square in (F). Note that the cell wall materials were deposited between the torn cuticles (black arrow). Cu, cuticle; CW, cell wall; V, vacuole. Bars = 1 μm (A, C and F) and 300 nm (B, D, E and G).

this activity was reduced to <10% of the maximum activity by heating for 10 min at 99°C (**Fig. 5B**). The optimum pH of this enzyme was broad, in the range of pH 7.0–9.0, and activity slightly decreased at acidic pH values (**Fig. 5C**). The specific activity was determined to be 45.0 μmol min^{-1} mg^{-1} CDEF1 protein.

CDEF1 is expressed in pollen, pollen tubes and the zone of emergence of lateral roots

To analyze the expression pattern of *CDEF1*, we performed a promoter analysis using the GFP–CDEF1 fusion protein as a reporter. In this analysis, we used a 1,903 bp region upstream of ATG as a promoter and a 237 bp region downstream of the stop codon as a terminator. GFP signals were detected in mature pollen grains within non-dehiscent anthers (**Fig. 6A–C**) and in pollen tubes that were germinating on the stigma (**Fig. 6D–F**). Interestingly, a GFP signal was detected as a patchy pattern in primary roots (**Fig. 6G–I**) and in restricted regions of the primary root periderm, where lateral roots were emerging (**Fig. 6J–L**). These expression patterns may be correlated with the emergence of lateral roots. These results were consistent with the AtGENExpress developmental data, which showed that the *CDEF1* gene is highly expressed in mature pollen and is expressed at low levels in roots (**Supplementary Fig. S4**).

Discussion

CDEF1 is a possible cutinase

The genes of plant cutinases are unidentified, although a few studies have characterized plant cutinases in pollen (Shayk and Kolattukudy 1977, Maiti et al. 1979, Hiscock et al. 1994). In this study, we isolated CDEF1 in a functional analysis in which the ectopic expression of *CDEF1* was found to cause defects in the cuticle (**Figs. 1, 2**). The defective phenotype is the same as that caused by the ectopic expression of *Fusarium* cutinase in Arabidopsis (Sieber et al. 2000). Analysis of the localization of GFP-tagged CDEF1 showed that CDEF1 is an extracellular protein (**Fig. 3**), suggesting that CDEF1 directly attacks the extracellular cuticle of transgenic *35S::CDEF1* plants. Moreover, the recombinant CDEF1 protein exhibited esterase activity (**Fig. 5**). These observations suggest that the phenotype caused by the ectopic expression of CDEF1 is due to the direct degradation of

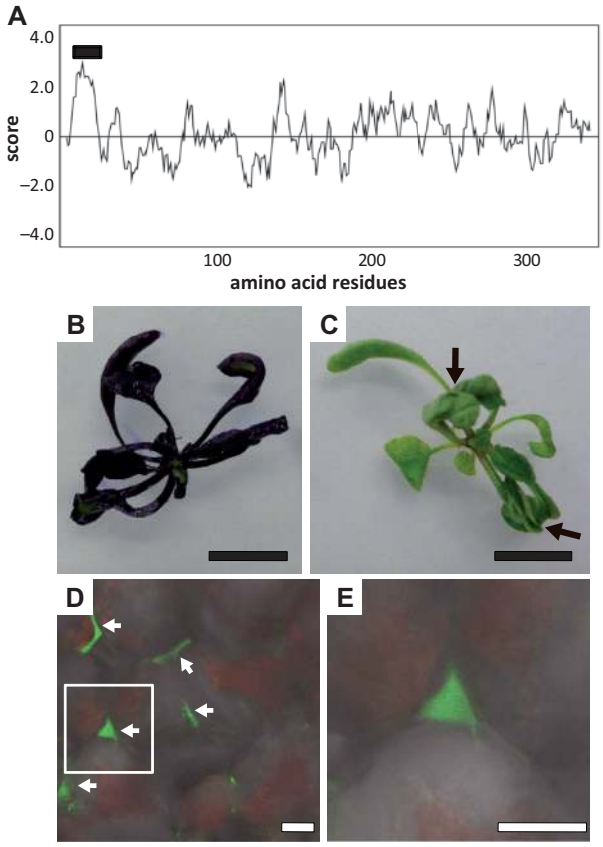

Fig. 3 CDEF1 is secreted into the extracellular space. (A) A hydropathy plot of CDEF1. CDEF1 has a hydrophobic region at its N-terminus (indicated by a gray bar). This is a putative signal peptide (SP) for incorporation into the secretory pathway. (B) and (C) transgenic *35S::SP-GFP-CDEF1* plants stained readily with TB (B), and occasionally exhibited the organ fusion phenotype (C). The fused leaves are indicated by black arrows. These phenotypes are the same as in transgenic *35S::CDEF1* plants. (D) Leaves of transgenic *35S::SP-GFP-CDEF1* plants were inspected with a confocal laser scanning microscope and a differential interference contrast (DIC) microscope. The merged image of the GFP (green), chloroplast autofluorescence (red) and DIC (gray) images is shown. GFP signals were detected in the extracellular space (white arrows). (E) Magnified view of the area enclosed by the white square in (D). Bars = 1 cm (B and C) and 10 μm (D and E).

the cuticle by CDEF1. Our findings support the possibility that CDEF1 is a plant cutinase.

CDEF1 belongs to a different gene family from fungal and bacterial cutinases

We found that the ectopic expression of selected genes of α/β hydrolase proteins did not cause cuticular defects. In the current plant genome and/or protein databases, there are no plant homologs of fungal and bacterial cutinases, all of which belong to the α/β hydrolase superfamily (Egmond and de Vlieg 2000, Chen et al. 2008). These observations imply the existence of plant cutinases that belong to protein families other than the α/β hydrolase superfamily.

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CDEF1     MVEGES KALWIILATV FAVAAVAPAV HGQQTPCYFV FGDSVFDNGN NNALNT--KA 54
GLIP1     MENSQLVSIT FLAYTIIISI GSINCIDNNN LVTNQSALFV FGDSVFDAGN NNYIDTLSSV 60

          KVNYLPYGID YFQGPTGRFS NGRNIPDVIA E---LAGFNN PIPPFAGASQ AQANIGLNYA 111
          RSNYWPYGQT TFKSPTGRVS DGRLIPDFIA EYAWLPLIPP NLQPFNGNSQ FAYGVNFASG 120

          SGAGGIREET SENMGERISL RQ--QVNNHF SAIITAAVPL SRLRQCLYTI NIGSNDYLNN 169
          GAGALVGTFS GLVINLRTQL NNFKKVEEML RSKLGDAEGK RVISRAVYLF HIGLNDYQYP 180

          YFLSPPTLAR RLFNPDQYAR SLISLYRIYL TQLYVLGARN VALFGIGKIG CTP-RIVATL 228
          -FTTNSSLFQ SI-SNEKYVD YVVGNMTDVF KEVYNLGGRK FGILNTGPYD CAPASLVIDQ 238

          GGGTGCAEEV NQAVIIFNTK ----LKALVT DFNNKPGAMF TYVDLFSGNA EDFAALGITV 284
          TKIRSCFQPV TELINMHNEK LLNGLRRLNH ELSGFKYALH DYHTSLSERM NDPSKYGFKE 298

          GDRSCCTVNP ---GEELCAA- -----NGPVC PDRNKFIFWD NVHTTEVINT VVANAAFNGP 336
          GKKACCGSGP LRGINTCGGR MGLSQSYELC ENVTDYLFFD PFHLTEKANR QIAELIWSGP 358

          ---IASPFNIS QLVN   348
          TNITGPYNLK ALFELN 374
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Fig. 4 Alignment of the deduced amino acid sequences of CDEF1 and GLIP1. Sequences were aligned using ClustalW. Bars above the sequences indicate the consensus regions of the GDSL lipase/esterase family of proteins. The invariant residues forming the catalytic triad and oxyanion hole are shown in red.

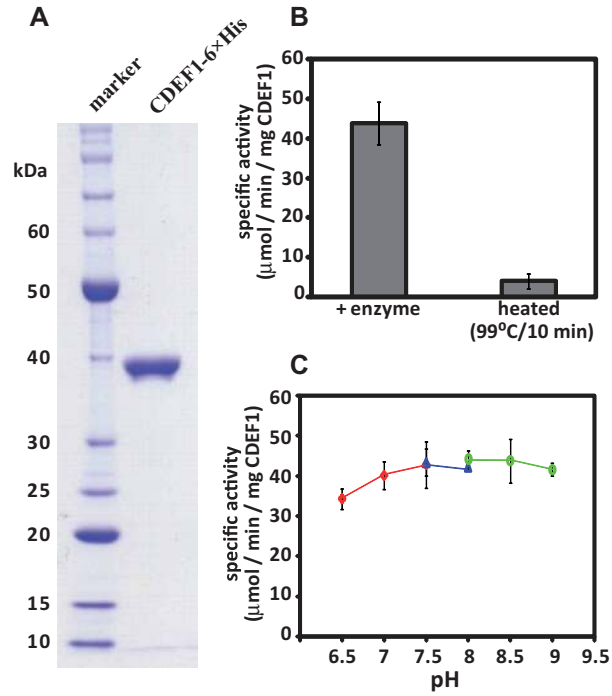

Fig. 5 Recombinant CDEF1 protein has esterase activity. (A) SDS–PAGE analysis of the purified recombinant CDEF1 (3 μg). The gel was stained with Coomassie Brilliant Blue to visualize proteins. (B) The esterase activity of the recombinant CDEF1. Assays were performed at 30°C in Tricine-NaOH buffer (pH 8.5) using *p*-nitrophenyl butyrate (pNPB) as the substrate. Esterase activity was abolished by heating the sample at 99°C for 10 min. Error bars indicate the standard deviation ($n = 3$). (C) The pH dependency of the enzyme activity was determined between pH 6.5 and pH 9.0, using pNPB as the substrate. The enzyme assay was performed at 30°C in each of the following buffers: Na-phosphate buffer (pH 6.5, 7.0, 7.5; red), HEPES-NaOH buffer (pH 7.5, 8.0; blue) and Tricine-NaOH buffer (pH 8.0, 8.5, 9.0; green). Error bars indicate the standard deviation ($n = 3$).

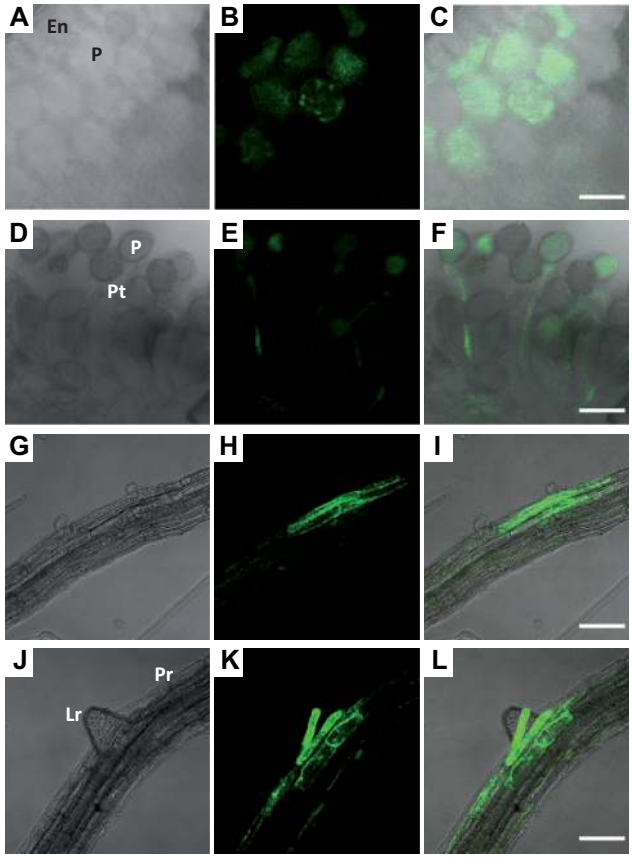

Fig. 6 *CDEF1* promoter activity was detected in pollen, pollen tubes and lateral root emergence zones. The *CDEF1 promoter*::*SP-GFP-CDEF1* construct with the *CDEF1* terminator was transformed into Col-0 plants. T_2 lines of transgenic plants were observed with a confocal laser scanning microscope and a differential interference contrast (DIC) microscope. The left panels (A, D, G and J) show DIC images. The center panels (B, E, H and K) show GFP fluorescence. The right panels (C, F, I and L) show the merged images. (A–C) GFP signals were detected in mature pollen in anthers prior to dehiscence. (D–F) GFP signals of pollen and pollen tubes on pollinated stigmas. (G–L) GFP signals were also detected in some restricted regions of the periderm of primary roots (G–I) and in the periderm of the primary roots around zones of lateral root emergence (J–L). En, endothecium; Lr, lateral root; P, pollen; Pt, pollen tube. Bars = 20 µm (C and F), 80 µm (I) and 100 µm (L).

We determined that CDEF1 belongs to the GDSL lipase/esterase protein family (**Fig. 4**). Although the GDSL lipase/esterase protein family has no sequence similarity to the α/β hydrolase superfamily, it has the ability to cleave a variety of esters, as do some members of the α/β hydrolase superfamily (Holmquist 2000, Akoh et al. 2004). It is possible that the GDSL lipase/esterase protein family includes cutinases. The observation that CDEF1 belongs to a different protein family from fungal and bacterial cutinases suggests that plant cutinases are evolutionarily different from fungal and bacterial cutinases.

Possible roles of CDEF1 in plants

The high level of expression of the *CDEF1* gene in pollen (**Supplementary Fig. S4**) implies that CDEF1 plays a role in plant reproduction. Previous studies have suggested that plant cutinases are expressed in pollen prior to pollination and are required for pollen tube penetration of the stigma (Hiscock et al. 2002). A possible role for CDEF1 is to degrade the stigma cuticle during pollination. Consistent with this prediction, *CDEF1* expression was observed in pollen before anther dehiscence and in pollen tubes on the stigma surface (**Fig. 6A–F**). Since the aerial parts of plant bodies are surrounded by cuticles, the cuticle must be degraded before the plant body can be penetrated. Fungi and bacteria also make use of their cutinases to penetrate and infect plant bodies.

Interestingly, *CDEF1* expression was detected in roots (**Fig. 6G–I**), suggesting that CDEF1 also plays a role in underground tissues. To our knowledge, plant cutinases have not been predicted to have any physiological function in roots. Our detailed analysis revealed that CDEF1 was strongly expressed in the periderm of primary roots near areas of lateral root emergence (**Fig. 6J–L**). This expression pattern suggests that CDEF1 degrades some components of the cell wall to facilitate lateral root emergence. In fact, several genes potentially involved in cell wall remodeling were reported to have the same expression pattern as that of *CDEF1* in roots (Neuteboom et al. 1999, Gonzalez-Carranza et al. 2007, Swarup et al. 2008, Ogawa et al. 2009, Peret et al. 2009). Suberin, which is mainly composed of aliphatic polyesters derived from fatty acid derivatives like cutin (Franke and Schreiber 2007, Pollard et al. 2008), is a possible substrate of CDEF1 in roots. Suberin is deposited in the cell wall of not only the endodermal Casparian strip but also the periderm, to protect roots from the environment. Excessive suberization was reported to be an obstacle in the emergence of lateral roots (Armstrong and Armstrong 2005). Therefore, an enzyme capable of degrading aliphatic polyesters, such as cutinase, may play a role in lateral root emergence.

To clarify in vivo function of *CDEF1*, we established a *CDEF1*-deficient mutant (SALK_014093) that carries a T-DNA insertion in the coding region of *CDEF1* (**Supplementary Fig. S5A**). However, we could not find any abnormal phenotypes of the mutant such as reduced fertility (**Supplementary Fig. S5C, Supplementary Table S1**) or reduced lateral root emergence (**Supplementary Fig. S5E**). In the Arabidopsis genome, there exist three genes that encode proteins similar to CDEF1. One possible explanation is that other enzymes having cutinase activity may function redundantly. Further analysis is necessary to elucidate the function of plant cutinases.

Materials and Methods

Plant materials and growth conditions

Arabidopsis thaliana ecotype Columbia-0 (Col-0) plants were used as the wild-type plants and parent lines for all the transgenic plants established in this study. Seeds were surface sterilized with

70% ethanol and then sown onto 0.5% (w/v) Gellan Gum or 0.9% (w/v) agar plates containing 1×Murashige–Skoog (MS) salts, 1% sucrose, and 0.5% (w/v) MES-KOH buffer (pH 5.7). Seeds were germinated and grown at 22°C under continuous light (100 μE $m^{-2} s^{-1}$) in a growth chamber. When necessary, the seedlings were transferred onto vermiculite for subsequent growth before the floral stem appeared.

Cloning, plasmid construction and transformation

For vector construction, we sequenced at least eight independent clones of each amplified fragment. We finally picked up one clone that has the same sequence as the genome database.

The coding regions of *At2g05850*, *At2g19050*, *At3g52000*, *At3g54240*, *At3g55180* and *At4g30140* were amplified by PCR with cDNA from flowers of Col-0 plants. The following primers were used for cloning of each gene: for *At2g05850*, 5′-caccatgggaaaacaacaagattggtccgt-3′ (forward) and 5′-tcagggtgtttgaggagaggagtg-3′ (reverse); for *At2g19050*, 5′-caccatggccgaggcaatattcaa-3′ (forward) and 5′-ctataatcttgcaagccaag-3′ (reverse); for *At3g52000*, 5′-caccatgggaaaacgacaagattggtctgt-3′ (forward) and 5′-tcaggctgtttgagggagtggagtg-3′ (reverse); for *At3g54240*, 5′-caccatgaagaaatggttaataacaggagc-3′ (forward) and 5′-ttaagatgattgaggaataggcaagag-3′ (reverse); for *At3g55180*, 5′-caccatggttatgtacaaagaggattatgt-3′ and 5′-tcatttttgcatagaaaaaccatcatctac-3′ (reverse);for*At4g30140*(*CDEF1*),5′-caccatggtcgagggagagtccaa-3′ (forward) and 5′-ttaattcactaactgggatatgttgaacgg-3′ (reverse). The fragment encoding *SP-GFP-*(*ΔSP*)*At4g30140* was generated by overlapping PCR, as follows. In the first PCR, two fragments encoding *SP-GFP* and *At4g30140* without the SP [(*ΔSP*)*At4g30140*] were amplified separately. These fragments were then fused in a second PCR. The following primers were used for the overlapping PCR: for *SP-GFP*, 5′-caccatggccagactcacaagcat-3′ (forward) and 5′-gcgtttgctgtccgccaccgagatctccct-3′ (reverse); for (*ΔSP*)*At4g30140*, 5′-cggtggcggacagcaaacgccgtgttactt-3′ (forward) and 5′-ttaattcactaactgggatatgttgaacgg-3′ (reverse); for the second PCR, 5′-caccatggccagactcacaagcat-3′ (forward) and 5′-ttaattcactaactgggatatgttgaacgg-3′ (reverse). The amplified fragments were inserted into pENTER/D-TOPO (Invitrogen, Carlsbad, CA, USA) to produce the entry clones. We performed L/R recombination reactions to transfer the fragments of the entry clones into the destination vector pH2GW7 or pB2GW7 (Karimi et al. 2002) for expression driven by the 35S promoter.

The *At4g30140* promoter (−1,903 to −1 bp upstream of *At4g30140*) and terminator (+1 to +237 bp downstream of *At4g30140*) were amplified by PCR from genomic DNA of Col-0 plants. We performed overlapping PCRs to generate the fragment encoding *At4g30140 promoter*::*SP-GFP-*(*ΔSP*)*At4g30140*:*At4g30140 terminator*. The *At4g30140 promoter*, *SP-GFP-*(*ΔSP*)*At4g30140* fragment and *At4g30140 terminator* were amplified in the first PCR. Then, the three PCR products were fused in a second PCR. The following primers were used for the overlapping PCR: for *At4g30140 promoter*, 5′-ccggaattcccaccctttcaagttccaaaattgg-3′ (forward) and 5′-gtctggccattccctcgaccatttttcgca-3′ (reverse); for *SP-GFP-*(*ΔSP*)*At4g30140*, 5′-ggtcgagggaatggccagactcacaagcat-3′ (forward) and 5′-ttaattcactaactggatatgttgaacgg-3′ (reverse); for *At4g30140 terminator*, 5′-aattagggctctattccattg-3′ (forward) and 5′-ccggaattccaactttgtatatagaatcggcaag-3′ (reverse); for the second PCR, 5′-ccggaattcccaccctttcaagttccaaaattgg-3′ (forward) and 5′-ccggaattccaactttgtatatagaatcggcaag-3′ (reverse). The amplified fragment and pENTER 1A (Invitrogen) were digested at the *Eco*RI site and ligated using a DNA Ligation Kit ver. 2 (TAKARA BIO INC., Shiga, Japan) to produce an entry clone. The entry clone fragment was transferred into the destination vector pHGW (Karimi et al. 2002) by the L/R reaction.

The expression vectors were introduced into *Agrobacterium tumefaciens* (strain GV3101) by electroporation. Wild-type plants were transformed with the *A. tumefaciens* by the floral dip method (Clough and Bent 1998). T_1 seeds were selected in a medium containing 25 mg l^{-1} hygromycin (for T_1 seeds carrying pHGW or pH2GW7) or 15 mg l^{-1} BASTA (for T_1 seeds carrying pB2GW7) to establish independent transgenic lines.

Online database, microarray data and software for reverse genetic analysis

The deduced coding sequence of each gene and the promoter sequence of *At4g30140* were obtained from The Arabidopsis Information Resource (TAIR). The lists of annotated genes from the α/β hydrolase superfamily (SUPERFAMILY: SSF53474) and GDSL lipase/esterase family (Pfam: PF00657) were obtained using the Bulk Data Retrieval tool in TAIR. We used the developmental data of AtGenExpress (Schmid et al. 2005) to analyze the tissue specificity of gene expression. Heat maps of the expression patterns were made using the TIGR MultiExperiment Viewer (MEV) v3.1.

The toluidine blue test

Aerial parts of plants were stained at room temperature for 30 s in 0.05% TB solution (Tanaka et al. 2004). The samples were rinsed with water before being photographed.

Fluorescence microscopy

To observe GFP fluorescence and chloroplast autofluorescence, we used a confocal laser scanning microscope (LSM 510 META; Carl Zeiss, Jena, Germany), with excitation wavelengths of 488 and 543 nm, emitted from a 40 mW Ar/Kr laser, and the appropriate emission filters (BP 505–530 and LP 505). We used an oil-immersion objective [Plan-Neofluar 100×1.3 numerical aperture (NA)] and dry objectives (40×0.95 NA, 20×0.80 NA, 10×0.50 NA) to view the samples.

RT–PCR

Total RNA was isolated from leaves of 21-day-old plants or flowers using an RNeasy Plant Mini Kit (QIAGEN, Valencia, CA, USA). Contaminating genomic DNA was digested by incubation with DNase I at 30°C for 15 min. DNase I was inactivated by the addition of EDTA and by incubation at 65°C for 30 min. A 200 ng aliquot of total RNA was subjected to first-strand cDNA synthesis using Ready-To-Go RT-PCR beads (GE Healthcare

Japan, Tokyo, Japan) with an oligo(dT)12–18 primer. PCR was performed using the cDNAs and ExTaq polymerase (TAKARA BIO INC.). The following primers were used for PCR: for *CDEF1*, 5′-atggtcgagggagagtccaaggc-3′ (forward) and 5′-gacgacgagctag agtaggaggcg-3′ (reverse); for *ACTIN*, 5′-ggcgatgaagctcaatccaaa cg-3′ (forward) and 5′-ggtcacgaccagcaagatcaagacg-3′ (reverse).

Expression and purification of recombinant CDEF1

Recombinant CDEF1 was expressed using the tomato mosaic virus infection system, essentially according to previous reports (Dohi et al. 2006, Dohi et al. 2008). Briefly, a saturated transgenic BY2 tobacco cell culture was diluted 1:100 in fresh medium, and β-estradiol was added to the culture to a final concentration of 10 μM at 2 d post-subculture. After a further 3 d incubation period, the cell culture was centrifuged for 3 min at 800 r.p.m. and the supernatant, containing secreted recombinant protein, was collected. The recombinant protein was purified and concentrated on an Ni^{2+} affinity column. The imidazole was then removed by dialysis.

Transmission electron microscopy

Leaves of wild-type and transgenic *35S::SP-GFP-CDEF1* plants were vacuum infiltrated with a fixative for 1 h, dehydrated, and embedded in Epon. Thin sections were stained with 4% uranyl acetate and lead citrate, and were examined with a transmission electron microscope (model 1200EX, JEOL).

Enzyme assays

Esterase activity was determined in a continuous spectrophotometric assay, using pNPB as a substrate. The activity was measured in a solution (200 μl) containing appropriate buffers (50 mM), pNPB (1 mM) and the enzyme (31.5 ng) at 30°C. The buffers used for each pH were Na-phosphate buffer (pH 6.5, 7.0, 7.5), HEPES-NaOH buffer (pH 7.5, 8.0) and Tricine-NaOH buffer (pH 8.0, 8.5, 9.0). The hydrolysis of pNPB was spectrophotometrically monitored for the formation of *p*-nitrophenol at 405 nm.

T-DNA insertion mutant

We obtained a T-DNA insertion line (SALK_014093) which carries a T-DNA insertion in the coding region of *CDEF1* from the ABRC (Arabidopsis Biological Resource Center) at Ohio State University. We isolated a homozygous mutant line by genotyping and designated it as *cdef1*. The following primers were used for PCR-based genotyping: for detection of the intact *CDEF1* gene, 5′-ttgttttggatcggattcaag-3′ (forward) and 5′-aaattacatgacaattcttcagcttaac-3′ (reverse); and for detection of the T-DNA insertion in the *CDEF1* gene, 5′-tggttcacgtagt gggccatcg-3′ (forward) and 5′-aaattacatgacaattcttcagcttaac-3′ (reverse).

Supplementary data

Supplementary data are available at PCP online.

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