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A subtilisin-like serine protease is required for epidermal surface formation in *Arabidopsis* embryos and juvenile plants

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SUMMARY

The surfaces of land plants are covered with a cuticle that is essential for retention of water. Epidermal surfaces of *Arabidopsis thaliana* embryos and juvenile plants that were homozygous for *abnormal leaf shape1* (*ale1*) mutations were defective, resulting in excessive water loss and organ fusion in young plants. In *ale1* embryos, the cuticle was rudimentary and remnants of the endosperm remained attached to developing embryos. Juvenile plants had a similar abnormal cuticle. The *ALE1* gene was isolated using a transposon-tagged allele *ale1-1*. The predicted ALE1 amino acid sequence was homologous to those of subtilisin-like serine proteases. The *ALE1* gene was found to be expressed within certain endosperm cells adjacent to the embryo and within the young embryo. Expression was not detected after germination. Our results suggest that the putative protease ALE1 affects the formation of cuticle on embryos and juvenile plants and that an appropriate cuticle is required for separation of the endosperm from the embryo and for prevention of organ fusion.

Key words: *Arabidopsis thaliana*, Epidermal differentiation, Cuticle, Subtilisin-like serine protease, Endosperm, *ABNORMAL LEAF SHAPE* (*ALE1*), Leaf morphology

INTRODUCTION

The outer surfaces of land plants and animals are characterized by an epidermis, which may produce an exocytoskeletal 'cuticle' or epidermal cytoskeleton that plays a protective role. Various components and structures are utilized by different organisms for this purpose. For example, the major structural components of the plant cuticle, which was originally isolated as a transparent thin film from cabbage leaves (Brongniart, 1834), are derivatives of lipids (cutins) (Baker and Martin, 1963; Kolattukudy, 1970; Martin and Juniper, 1970; Kolattukudy, 1980a; Kolattukudy, 1980b; Holloway, 1982), whereas in *Caenorhabditis elegans* they are proteins (cuticle collagens) [reviewed by Johnstone (Johnstone, 2000)].

In *C. elegans*, mutations in genes for several cuticular collagens, for factors involved in X-chromosome dosage compensation, and for a subtilisin-like serine protease result in deformed gross morphology and altered cuticle formation and/or function (Johnstone, 2000; Thacker et al., 1995). In vertebrates, several factors that mediate extracellular signals, such as bone morphogenetic proteins (BMPs), their activators that are subtilisin-like serine proteases (called subtilisin-related proprotein convertases in vertebrates) such as furin and/or PC6, and their receptors are involved in the normal differentiation of the epidermis (Graff, 1997; Cui et al., 1998).

The epidermis of higher plants plays important roles in development, water retention, defence against pathogens and gas exchange. In *Arabidopsis thaliana*, the epidermis is derived from an outer cell layer, or protoderm of the embryo proper that is formed in the 16-cell embryo proper stage (Goldberg et al., 1994). The environment surrounding the embryo changes dramatically when the seed germinates: the embryo, enclosed by the endosperm and seed coat during seed development, comes in contact with the outer environment for the first time. Thus, embryos must prepare for successful germination, the seed must sense suitable conditions to germinate, and the seedling must respond to changing environmental conditions (Dure III, 1975; Howell, 1998). For survival, the surface functions of the epidermis should be present, at least in part, during embryo development and, indeed, a cuticle layer is apparent on the surface of the developing *Arabidopsis* (Bowman and Mansfield, 1993; Rodkiewicz et al., 1994), *Citrus* (Bruck and Walker, 1985), maize (Van Lammeren, 1986), *Capsella* (Rodkiewicz et al., 1994) and *Stellaria* (Rodkiewicz et al., 1994) embryos. However, little is known about the genes involved in epidermal differentiation and/or epidermal function during embryogenesis in higher plants.

The *fiddlehead* (*fdh*) mutant and several other mutants of *A. thaliana* and maize are characterized by their striking postgenital organ fusion in leaves and/or flower organs (Lolle

et al., 1992; Sinha and Lynch, 1998; Lolle and Pruitt, 1999). In these mutants, organ fusion often, but not always, coincides with pollen germination and with alterations in the chlorophyll permeability barrier (Lolle et al., 1998), indicating that alteration in a fundamental property of the epidermal surface result in organ fusion. In the maize *crinkly4* mutant, striking changes occur in epidermal cell morphology and cells at the fused region appear to proliferate (Becraft et al., 1996). Epidermal cells of *cr4* mutants show irregularities in the structure of cell walls and the formation of cuticle (Jin et al., 2000). The *CR4* gene encodes a receptor protein kinase, the extracellular domain of which contains a cysteine-rich region that is similar to the ligand binding domain of tumor necrosis factor receptors (TNFRs) in mammals, suggesting that the *CR4* gene is involved in epidermal differentiation (Becraft et al., 1996). Recently, transgenic *Arabidopsis* plants that expressed a fungal cutinase have been reported to exhibit organ fusion. Therefore, an intact cutin layer not only is important for plant-environment interactions but also prevents fusions between different plant organs (Sieber et al., 2000).

We report the isolation of a novel *Arabidopsis* mutant named *abnormal leaf shape1(ale1)*, which results in defects in cuticle formation and the surface function of embryos and seedlings. The *ale1* embryos were covered by rudimentary cuticle and adhered to endosperm (extra embryonic tissue). The mutant seedlings were sensitive to decreasing humidity and eventually dried up. Under conditions of high humidity, they were able to produce leaves, which were fused to cotyledons and/or the other leaves. We isolated the *ALE1* gene and show that it encodes a novel member of the subtilisin-like serine protease family, members of which are also involved in proper epidermal differentiation in animals. Many members of this family of proteases were reported in plants (Kaneda et al., 1984; Kobayashi et al., 1994; Yamagata et al., 1994; Ribeiro et al., 1995; Rundenskaya et al., 1995; Tornero et al., 1996a; Tornero et al., 1997; Rundenskaya et al., 1998; Bogacheva et al., 1999; Jorda et al., 1999; Meichtry et al., 1999; Neuteboom et al., 1999; Batchelor et al., 2000; Berger and Altmann, 2000; Jorda et al., 2000; Laplaze et al., 2000; Yamagata et al., 2000) and some of them were shown to have protease activities in vitro (Kaneda and Tominaga, 1975; Vera and Conejero, 1988; Rundenskaya et al., 1995; Rundenskaya et al., 1998). However, little is known about the role of the subtilisin-like serine proteases of plants except for the *SDD1* gene which was found to be involved in regulation of stomatal density and distribution (Berger and Altmann, 2000). The *ALE1* gene is specifically expressed during seed development. In situ hybridization experiments showed that the *ALE1* transcript accumulates strongly in endosperm cells and weakly in young embryo. These results indicate that endosperm is involved in the formation of a proper surface of the developing embryo.

MATERIALS AND METHODS

Plants and growth conditions

Arabidopsis thaliana ecotype Landsberg *erecta* (L*er*) was used as the wild type. The *ale1-1* plant was isolated from a dAc-I-RS-tagged line that contained a dAc-I-RS (a derivative of *Ac*), and a gene for *Ac* transposase on separate chromosomal locations (Machida et al., 1997). The *ale1-1* plant was backcrossed at least once to segregate the transposase gene and stabilise the dAc-I-RS before analyses of phenotypes. An M_2 population of L*er* seeds mutagenized by ethyl methanesulfonate (EMS) was purchased from Lehle Seeds (Round Rock, TX, USA), and we isolated the *ale1-2* plant. The *ale1-2* plant was backcrossed three times.

For analyses of seedling phenotypes, vernalization treatments were performed to facilitate coordinated germination. Unless otherwise noted, seeds were sown on soil in containers and the containers were covered with transparent plastic film and stored for 2 days at 4°C. They were then transferred to a growth chamber (22°C, 16-hour photoperiod) and uncovered 3 days after the vernalization treatment (DAV). For histological analysis of leaves and ultrastructural analysis of seedlings, we surface sterilized seeds and sowed them on Murashige and Skoog plates containing Murashige and Skoog salts (Wako Pure Chemical Industries, Ltd., Osaka, Japan). After the vernalization treatments, they were transferred to a growth chamber (22°C, continuous photoperiod).

Genetic analysis and cloning of the *ALE1* gene

No recombination between the transposon and the *ale1-1* mutation was observed during 160 independent meioses, suggesting that the *ale1* gene was tagged by dAc-I-RS. Flanking regions of the transposon were isolated by the inverse polymerase chain reaction (IPCR) and a genomic clone that contained the IPCR fragment was isolated, and sequenced. Four independent revertants (*ale1-1R1* through *ale1-1R4*) were obtained by screening for plants with morphologically normal cotyledons. Genomic DNA fragments containing the footprint sequences were PCR amplified and sequenced.

Microscopy

Plastic sections were prepared and observed as follows. Tissues were fixed in 4% paraformaldehyde, dehydrated in a graded ethanol series and mounted in Technovit 7100 (Heraeus Kulzer, Wehrheim, Germany) essentially as described in the instructions from the manufacturer. Sections of 3-5 μm were cut with a microtome (HM360, Carl Zeiss, Tokyo, Japan) with disposable blades (C35, Feather Safety Razor, Osaka, Japan), and stained with 0.05% Toluidine Blue. For observation of epidermal surfaces of cotyledons, samples were frozen in liquid nitrogen and observed with a scanning electron microscope (XL30; Philips Electron Optics, Tokyo, Japan). For transmission electron microscopic analysis, samples were processed as described previously (Nishimura et al., 1993).

cDNA cloning

Polyadenylated RNA was prepared from developing seeds. For reverse transcription PCR, first-strand cDNA was generated as described (Hamada et al., 2000). *ALE1* cDNA corresponding to the first exon to the tenth exon was amplified by PCR with primers ALE1-5′D3 (5′-CTAATGGAACCTAATCCAAG-3′) and ALE1-3′U3 (5′-GTTTTCAAATGGTTTTAACTGACAAC-3′). For 5′ rapid amplification of cDNA ends (RACE), first-strand cDNA was synthesized with the primer ALE1RTP (5′-CTGCCTTCACTCCTT-3′) and self-ligated. RACE was performed with primers A1_5′S1 (5′-GGAAGCACACTTGAAAAGGGA-3′) and A1_5′A1 (5′-GCTC-TGGACAATATTGCACA-3′), and nested PCR was performed with primers A1_5′S2 (5′-CTTTTGCAGTCCGTACCA-3′) and A1_5′A2 (5′-CATACTATCAGCCATATGTAGGA-3′) with a 5′ Full RACE Core Kit (TaKaRa, Tokyo, Japan) according to the manufacturer's protocols. 3′ RACE was performed with primer ALE1_3′1 (5′-CGCCACAGGAGTCTTGTGTACCACTACGCTTAGCCACCC-3′) and the SMART RACE cDNA Amplification Kit (Clontech, Tokyo, Japan) according to the manufacturer's protocol.

Expression analyses

RNA gel blot analysis was performed as described (Hamada et al.,

2000). RNA was isolated from the wild type and the stabilised *ale1-1* plants and 1 μg of poly(A)$^+$ RNA was electrophoresed. A 432 bp PCR product that corresponded to the last 112 codons and 3′ non-coding region was cloned into pBluescript SK– (Stratagene, La Jolla, CA, USA) to generate the pALE13RU. A *Bam*HI-*Xho*I fragment from pALE13RU was used to generate a hybridization probe. In situ hybridization was performed as described (Long and Barton, 1998; Semiatri et al., 2001). A 874 bp PCR product (nt 162 to nt 1035 of *ALE1* cDNA) that corresponded to the first to sixth exons was cloned into pBluescript SK– (Stratagene) to generate the pALE1151410 and pALE115146. Antisense RNA probes were generated by linearizing pALE1151410 with *Bam*HI and synthesized using T7 RNA polymerase. Sense control probes were synthesized using T7 RNA polymerase on pALE115146 linearized with *Bam*HI. Hybridization using a probe that covered a part of the tenth exon and 3′ untranslated region gave essentially the same result (not shown). To prepare the *ALE1* promoter-GUS gene fusion construct, we fused a 2.7 kb genomic DNA fragment that contained the 5′-upstream region and part of the first exon of the *ALE1* gene in-frame to the gene for β-glucuronidase (GUS). The fragment was cloned into the transformation vector pGAB2 (our unpublished data) and the construct was used to transform *A. thaliana* ecotype Columbia. The assay for GUS activity was performed as described elsewhere (Koizumi et al., 2000), with the exception that samples were immersed in a reaction mixture that had been supplemented with 100 μg/ml chloramphenicol and incubated for 3 days.

RESULTS

The *ale1* mutation results in defective epidermal function

The recessive *abnormal leaf shape1-1* (*ale1-1*) mutation was found to affect epidermal surface functions, with resultant conditional lethality of the *ale1-1* seedlings. Most mutant cotyledons dried up soon after the germination of seeds and this phenomenon was sensitive to humidity (Fig. 1B,C). When seeds were allowed to germinate at relatively high humidity in an enclosed container and were then exposed to the open air 3 days after germination, 84% (n=192) of *ale1-1* plants wilted, while only 3% (n=184) of wild-type plants wilted. The mutant seedlings survived when the plants were kept at high relative humidity (data not shown), suggesting that seedling lethality was caused by water loss.

Surviving *ale1-1* plants produced small, crinkled cotyledons and leaves that often were fused with each other (Fig. 1E,F). Transverse sections through the fused leaves revealed that graft-like fusions had occurred (Fig. 1I). The epidermal pavement cells of the crinkled leaves were deformed (Fig. 1K) and the number of trichomes (epidermal hairs) was slightly reduced (data not shown). Histological analysis indicated that the internal anatomy was relatively unaffected unless the organ was severely deformed (Fig. 1H). These defects were frequently observed at the vegetative stage, in particular within cotyledons and juvenile leaves before leaf 4 (Fig. 1F,L) (Hamada et al., 2000), but were rarely observed subsequently.

An *ale1-2* allele was identified by screening the M_2 population of EMS-mutagenized L*er* seeds for seedlings with the phenotype similar to that of *ale1-1*. Since the phenotype of *ale1-2* appeared to be indistinguishable from that of *ale1-1* (data not shown), we performed most of our detailed studies on *ale1-1*.

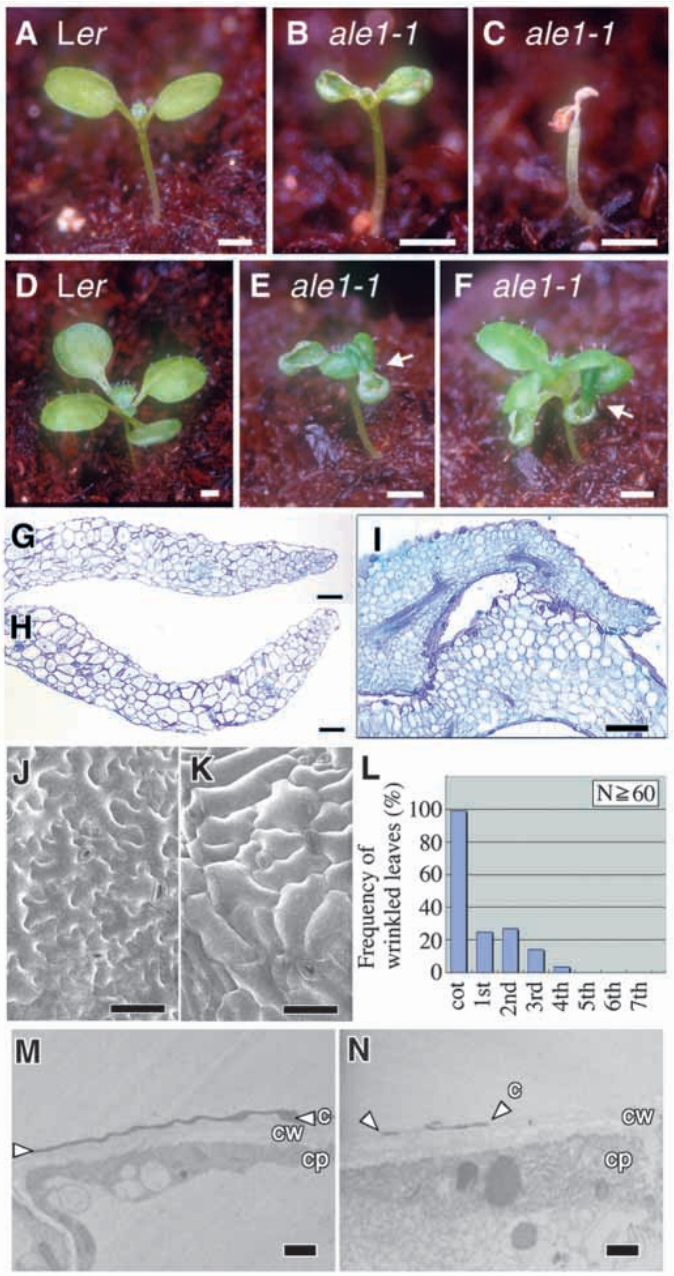

Fig. 1. The phenotype of *ale1* plants. (A-F) Gross morphology of aerial parts of plants. Plants were soil-grown in a covered container and the cover was removed 3 days after vernalization (DAV). (A) Wild-type plant, 7 DAV. (B,C) *ale1-1* plants, 7 DAV. (D) Wild-type plant, 12 DAV. (E) *ale1-1* plant, 12 DAV. (F) The same *ale1-1* plant shown in E, at 16 DAV. The arrows in E and F indicate fused organs. (G,H) Transverse sections of wild-type (G) and *ale1-1* (H) cotyledons. (I) Transverse section of fused leaves. (J,K) Adaxial surfaces of wild-type (J) and *ale1-1* (K) cotyledons, 7 DAV. (L) Frequency of wrinkled leaves at each leaf position. Morphology of cotyledons and true leaves were observed 1 week and 3 week after vernalization, respectively. (M,N) Transmission electron micrographs of epidermal surfaces of wild-type (M) and *ale1-1* (N) cotyledons. In the wild-type cotyledons, a densely staining layer covered the entire surface of each cotyledon. The arrowheads in M and N indicate the margin of the densely stained cuticle. cot, cotyledons; c, cuticle; cw, cell wall; cp, cytoplasm. Scale bars: 1 mm (A-F); 100 μm (G-I); 50 μm (J,K); and 1 μm (M,N).

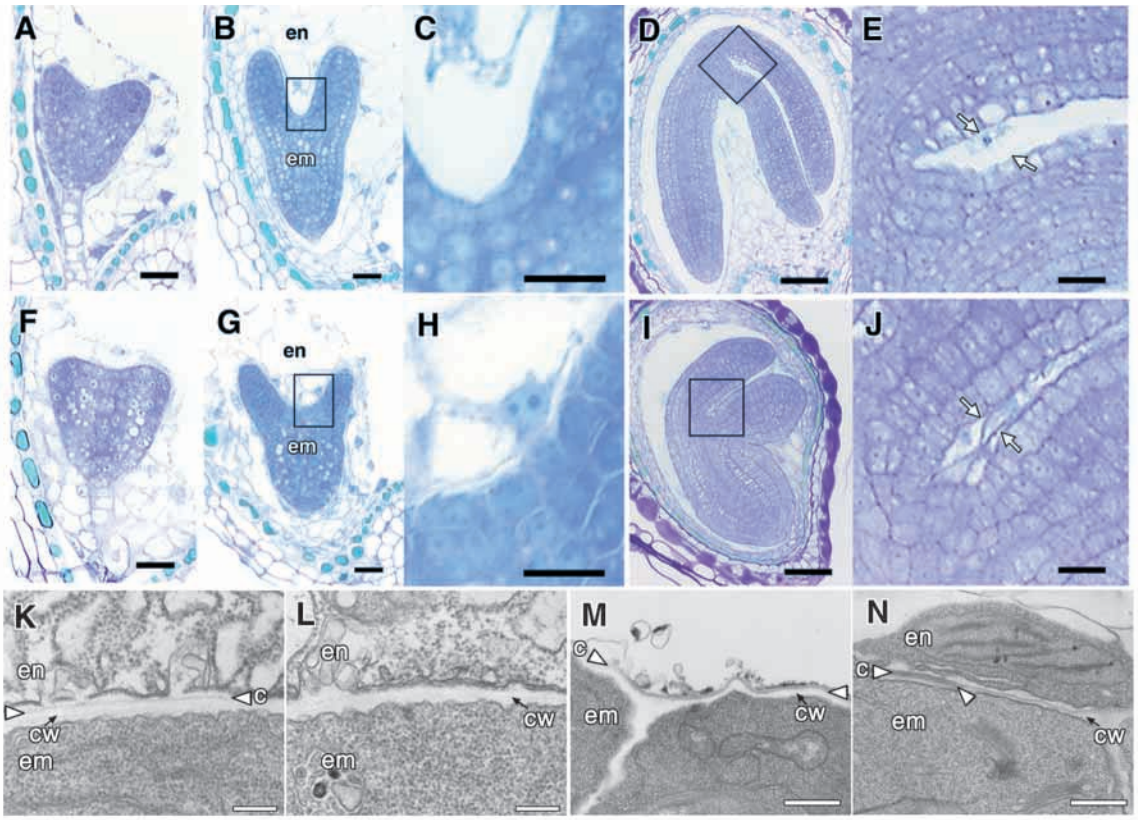

Fig. 2. Defects in *ale1* mutants in cuticle formation and adhesion of the endosperm to the embryo's surface. (A-E,K,M) Sections of wild-type seeds. (F-J,L,N) *ale1-1* seeds. At each stage, seeds were sectioned and observed by light microscopy (A-J) or transmission electron microscopy (K-N). (A,F) Early heart-stage embryos. (B and G) Torpedo-stage embryos. (C,H) Magnified views of the boxed areas in B and G, respectively. (D,I) Walking-stick stage embryos. (E,J) Magnified views of the boxed areas in D and I, respectively. Arrows indicate adaxial surfaces of cotyledons. (K,L) Surfaces of embryos at the 16- to 32-cell embryo proper stage. The arrowheads indicate the thin electron-dense layer in the outermost region of the epidermal cell walls of the embryo. (M,N) Surfaces of heart-stage embryos. A continuous densely stained cuticle covered the entire wild-type embryo (M). The cuticle on the surface of the *ale1-1* embryo was discontinuous (N; the arrowheads indicates the margin of the densely stained cuticle). em, embryo; en, endosperm; c, cuticle; cw, cell wall. Scale bars: 20 μm (A,B,F,G); 10 μm (C,E,H,J); 50 μm (D,I); 200 nm (K,L); and 500 nm (M,N).

Cuticle is rudimentary in *ale1* embryos and seedling

Since the epidermal cuticle plays important roles in water retention (Martin and Juniper, 1970; Kolattukudy, 1980a), we postulated that *ale1* plants might be defective in the formation of a functional cuticle. To confirm this possibility, we examined the epidermal surfaces of cotyledons by transmission electron microscopy. In embryos and leaves of wild-type plants, the cuticle can be detected as a thin electron-dense layer (Sterk et al., 1991; Sieber et al., 2000). We found that *ale1-1* cotyledons lacked the continuous layer of electron-dense materials (Fig. 1N, arrowheads) that generally covered the epidermal surface of wild-type cotyledons (Fig. 1M).

We also examined the surface of embryos, since a cuticle is formed during embryogenesis (Sterk et al., 1991). During embryogenesis, the embryo and endosperm undergo dynamic changes (Bowman and Mansfield, 1993). At the globular stage, the wild-type embryo was surrounded by a syncytial endosperm whose boundary was visible as a thin electron-dense layer in the outermost region of the epidermal cell walls of the embryo (Fig. 2K, arrowheads). The endosperm adheres to the embryo until the late globular stage, when a thicker, densely staining layer is visible at the embryo's periphery (Bowman and Mansfield, 1993). At the early heart stage, we were able to detect the start of cellularization of the endosperm at the mycropylar end (Fig. 2A). While cellularization continued toward the chalazal end, the endosperm started to degenerate and to become detached from the wild-type embryo (Fig. 2B,C), which was covered with a densely staining cuticle layer (Fig. 2M) (Bowman and Mansfield, 1993). At the walking-stick stage, the endosperm was almost completely separated from the embryo (Fig. 2D,E).

In *ale1-1* homozygous seeds, the boundary between the embryo and the endosperm was unclear at the globular stage (Fig. 2L). The mutant embryos and endosperm were morphologically indistinguishable from those of the wild type until the early heart stage (Fig. 2A,F). At the heart stage, the mutant embryo was covered by a discontinuous cuticle (Fig. 2N, arrowheads) and cellularized endosperm adhered to the embryo (Fig. 2N). The endosperm remained attached to the embryo at the torpedo stage (Fig. 2G,H), at the walking-stick stage (Fig. 2I,J), and even after desiccation and seed germination (not shown). When wild-type embryos were at the torpedo stage, gross morphology of *ale1-1* embryos was dumpy (short in the longitudinal axis and fat in the radial axis)

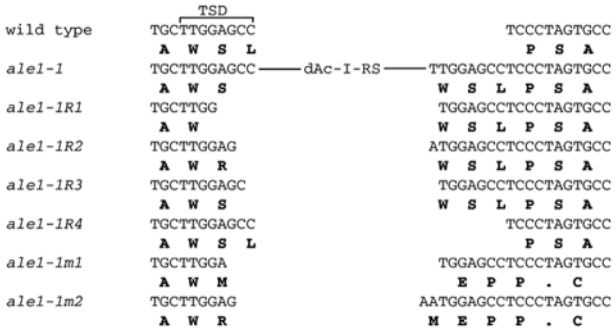

Fig. 3. Footprint alleles generated after excision of dAc-I-RS from the insertion allele *ale1-1*. The 8 bp duplicated upon dAc-I-RS insertion is labelled TSD. Footprints that leave the open reading frame intact (*ale1-1-R1* to *R4*) lead to reversions to wild-type, while plants carrying the footprints that disrupt the open reading frame (*ale1-1m1* and *m2*) are mutant.

and their cotyledons were not aligned in parallel (Fig. 2B,G). Thus, the earliest defect in the *ale1-1* mutant was the impaired formation of a densely staining layer at the embryo's surface. Later defects, such as endosperm adhesion, organ fusions and excessive water loss from *ale1* seedlings, might have been caused by the defect in cuticle formation on the epidermis of *ale1* embryos and juvenile plants.

ALE1 encodes a subtilisin-like serine protease

Because the *ale1-1* mutant was isolated from a population of transgenic plants containing transposable element dAc-I-RS (a derivative of *Ac*) (Machida et al., 1997), the *ALE1* gene could be tagged by the transposon. DNA gel blot hybridization using a dAc-I-RS sequence showed that the *ale1-1* plant contained a single copy of the dAc-I-RS sequence (data not shown). We cloned the flanking genomic sequence by inverse PCR. Linkage analysis detected no recombination between the insertion and the *ale1-1* mutation among 80 F_2 plants.

As shown in Fig. 3, four independent revertants (*ale1-1R1* through *R4*) were obtained from approx. 3,000 *ale1-1* seedlings. Each revertant was resistant to low humidity and produced morphologically normal leaves. PCR and sequence analyses showed that the wild-type plants contained revertant alleles that had lost the dAc-I-RS insert, indicating that the phenotypic reversion was due to the excision of dAc-I-RS. Three revertant alleles showed that the dAc-I-RS excision had generated a 3 bp (*R1*) and two 6 bp footprints (*R2* and *R3*) that leave the open reading frame intact (Fig. 3). In one revertant (*R4*), the excision occurred without any footprint. Taken together, these data show that the transposon insertion and excision in the genomic fragment fully correlate with phenotypic changes. Thus, this genomic fragment contains a part of the *ale1-1* gene. Southern hybridization using the genomic fragment as probe also

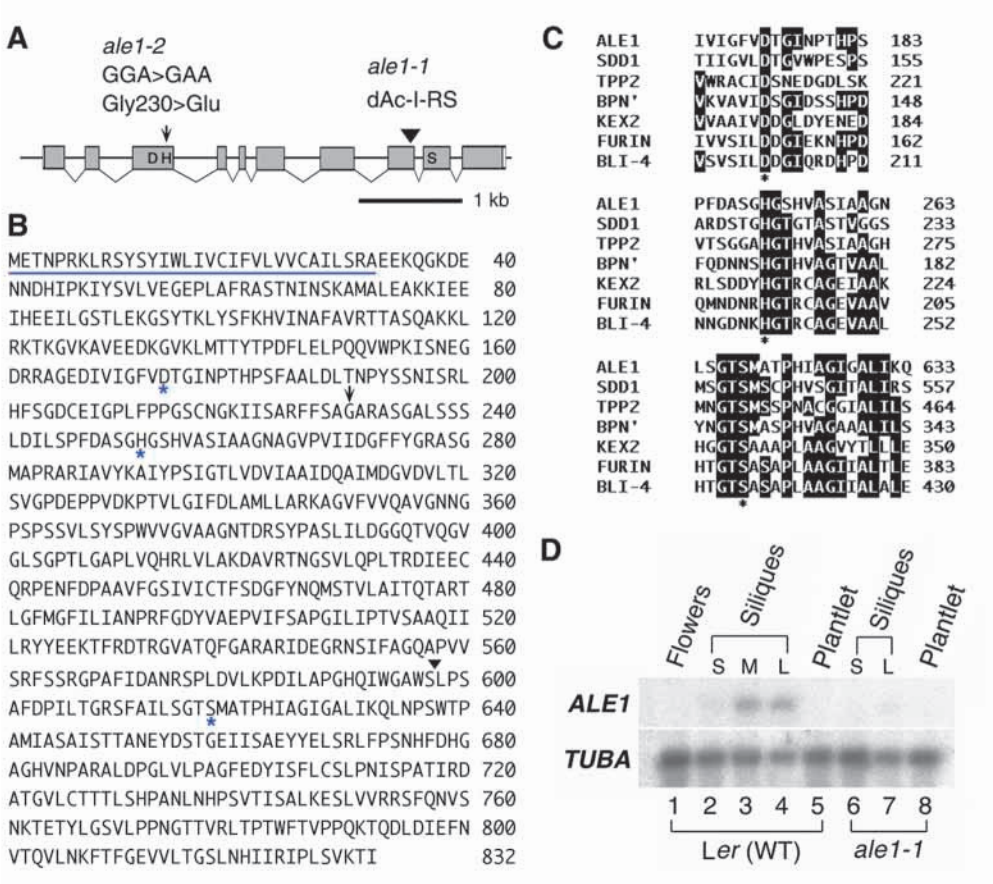

Fig. 4. Structure and patterns of expression of the *ALE1* gene. (A) Schematic diagram of the *ALE1* gene. White boxes, untranslated exons; gray boxes, translated regions. Positions of three amino acid residues (D, H and S) that are consistently conserved in the catalytic regions of the subtilisin-like serine proteases (the catalytic triad) are indicated (see B,C). The relative positions of the *ale1* mutations are shown. (B) Predicted amino acid sequence of the ALE1 protein (accession number: AB060809). A putative signal peptide is underlined. Three residues in the catalytic triad are indicated by asterisks in B and C. (C) Alignments of amino acid sequences around the catalytic triad of subtilisin-like serine proteases. SDD1, *A. thaliana*; TPP2, human tripeptidyl peptidase 2; BPN', *Bacillus amyloliquefaciens*, subtilisin BPN'; KEX2, *Saccharomyces cerevisiae*; FURIN, human; BLI-4, *C. elegans* (GenBank accession numbers: T00962, P29144, P00782, KXBY, P09958, and P51559, respectively). (D) The RNA on a gel blot prepared with poly(A)$^+$ RNA from flowers, siliques at various developmental stages, and young plants (plantlets; 12 DAV) was allowed to hybridize with an *ALE1* probe that covered part of the tenth exon and the 3′ untranslated region. As a control, the washed membrane was subsequently hybridized with a probe for α-tubulin (*TUBA*) (lower panel). S, Small siliques 2-4 days after pollination (DAP); M, medium siliques 3-6 DAP; L, large siliques 5-10 DAP.

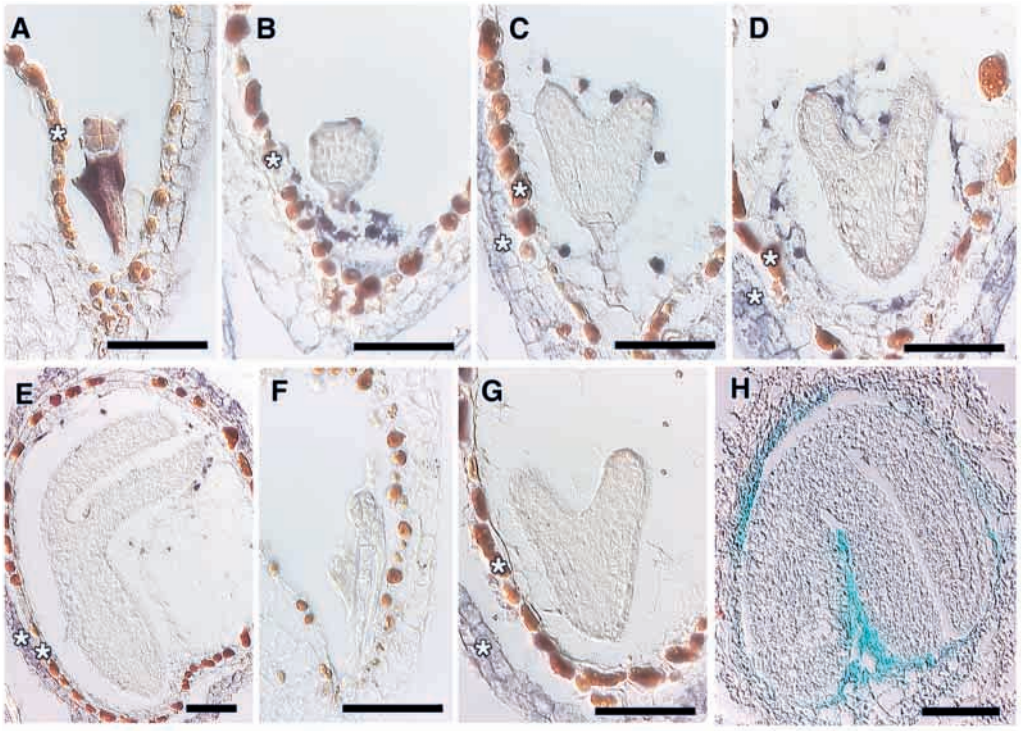

Fig. 5. *ALE1* is expressed in endosperm surrounding developing embryos. (A-E) Pattern of distribution of *ALE1* mRNA. (A) Seed containing the globular stage embryo. Signals (brown coloration) were observed in the endosperm around suspensor cells and weak signals were also detected in the embryo and suspensor cells themselves. (B) A globular-heart transition stage embryo and endosperm before cellularization. (C) Seed containing a heart-shaped embryo and cellularized endosperm. Strong signals (dark blue coloration) were observed in some of the cellularized endosperm that surrounded the embryo. (D) An embryo and endosperm at the heart stage. Strong signals were detected in some of the endosperm cells around the embryo. (E) Embryo and endosperm at the walking-stick stage. Signals were detected in some of the endosperm cells at positions closest to the embryo. (F,G) Sense controls for A to E. Reddish brown or weak blue signals in the cell layer underneath the seed coat and in the seed coat cells (indicated by white asterisks) were not specific to *ALE1* RNA because they were generated by both the antisense and the sense probe. (H) GUS activity in endosperm cells of a transgenic plant that harbored the *ALE1* promoter-GUS fusion gene. Scale bars, 50 μm.

suggested that *ALE1* is a single copy gene in *A. thaliana* (data not shown).

To determine the structure of the *ALE1* gene, we isolated *ALE1* cDNAs and corresponding genomic clones from wild-type, *ale1-1* and *ale1-2* plants, and determined nucleotide sequences (Fig. 4A,B). The *ale1-1* mutation was caused by insertion of dAc-I-RS into the eighth exon (Fig. 4A,B). The *ale1-2* mutation was mapped to the third exon; it resulted in a single amino acid substitution (Fig. 4A,B).

The predicted sequence of the ALE1 protein resembled those of subtilisin-like serine proteases such as the yeast endoprotease KEX2 required for production of a mating pheromone called alpha factor (Julius et al., 1984). The amino acid sequences around the three residues that are essential for the catalytic activity of subtilisin-like serine proteases (the catalytic triad) were conserved in all members of the family, including ALE1 (Fig. 4C). Moreover, like many other members of this family of proteases, the predicted amino acid sequence of ALE1 includes a putative signal sequence for secretion (von Heijne, 1986) at the amino terminus (Fig. 4B). Since the dAc-I-RS sequence was found in the coding region upstream of the catalytic serine residue of the putative ALE1 serine protease, the *ale1-1* seems to be a null allele. The *ale1-2* mutation resulted in an amino acid substitution of the glycine residue at position 230 to the glutamate residue. Because the *ale1-1* and the *ale1-2* are phenotypically indistinguishable, this residue may be critical for the function of ALE1. Supporting this notion, the glycine residue was present between the catalytic aspartate and histidine residues and conserved in most of subtilisin-like serine proteases from plants that have been reported (23/28 proteases). These results suggest that activity of the putative ALE1 protease is required for proper differentiation of epidermis.

ALE1 is expressed during seed development

To investigate the distribution of *ALE1* mRNA, we examined the hybridization of an *ALE1* probe to an RNA gel blot prepared with mRNAs from various tissues, including flowers, developing siliques and young plants. Signals were obtained in lanes loaded with RNA from developing siliques (Fig. 4D: lanes 2, 3 and 4) but not in those loaded with RNA from flowers and young plants. The band in lanes containing RNA from developing siliques of *ale1-1* plants was very weak and different in size from those of the wild-type plants (Fig. 4D: lanes 6 and 7). These results show that the hybridization signals in wild type were specific to *ALE1* mRNA.

To identify sites of accumulation of *ALE1* mRNA in developing siliques, we performed RNA in situ hybridization. Strong signals were evident at the globular stage and signals were even stronger in the endosperm that surrounded the embryo (Fig. 5A). The pattern of accumulation of *ALE1* RNA changed gradually from the globular stage to the heart stage, when the RNA was restricted to the endosperm cells that surrounded the embryo (Fig. 5B,C). After degradation of the endosperm had begun, signals were appreciable within endosperm cells located close to the embryo, which might have been in the process of being or about to be degraded (Fig. 5D,E). Consistent with these observations, β-glucuronidase (GUS) activity was detected in the endosperm but not in the mature embryos of transgenic plants that harbored an *ALE1* promoter-GUS reporter gene (Fig. 5H).

DISCUSSION

ALE1 promotes epidermal differentiation

We described that *Arabidopsis ale1-1* plants lack a continuous cuticle layer on the developing embryos and seedlings, leading to adhesion of endosperm to the embryo, conditional lethality of seedlings at decreasing humidity, irregular morphology of epidermal cells, and fusions between lateral organs. To our knowledge, this is the first report of a genetic link between incomplete cuticle formation, endosperm adhesion and the conditional lethality of seedlings. Since the *ale1-1* was a loss-of-function mutation, the wild-type *ALE1* gene is required for proper differentiation of epidermis.

Organ fusions have been reported in several mutant and transgenic plants (Lolle and Pruitt, 1999; Sieber et al., 2000). Among them, the *bulkhead* (*bud*) mutation maps to the bottom of chromosome 1 (Lolle et al., 1998), which might be close to the position of *ALE1* gene. According to Lolle et al., the *bud* mutant is recessive and shows defects only in reproductive organs. Therefore, the *ale1* is probably a novel mutation. Organ fusions were observed in *A. thaliana* expressing a fungal cutinase, indicating that loss of cuticle leads to organ fusion (Sieber et al., 2000), which is consistent to the idea that organ fusions in *ale1* plants might be caused by incomplete formation of a cuticle. The *fiddlehead* (*fdh*) mutant of *A. thaliana* also produces fused lateral organs. The *FDH* gene encodes a protein that is similar to the condensing enzymes involved in lipid biosynthesis (Yephremov et al., 1999; Pruitt et al., 2000) and the gene is expressed in the epidermis of young vegetative and floral organs. Since the *FDH* gene might be involved in cuticle formation, we examined whether *FDH* was expressed normally in *ale1* embryos. We monitored the expression of *FDH* mRNA in wild-type and *ale1-1* embryos by in situ hybridization but failed to find any difference in levels of *FDH* mRNA (data not shown). We also examined the expression of several genes that are specifically expressed in the protoderm of the embryo [e.g., *ATML1*, *CUT1*, *PDF1* (Lu et al., 1996; Millar et al., 1999; Abe et al., 1999)]. However, we again found no differences between wild-type and *ale1-1* embryos (data not shown). Expression patterns of *ATML1* and *PDF1* were essentially identical to those reported previously (Lu et al., 1996; Abe et al., 1999). Our results showed that expression patterns of *FDH* and *CUT1* were similar to those of *ATML1* and *PDF1* (our unpublished data). Our observations suggest that neither *ALE1* nor separation of the endosperm is required for the expression of these genes in the protoderm. ALE1 controls very specific aspects of epidermal differentiation, perhaps only cuticle formation.

Endosperm function in surface formation of developing embryo

Since *ALE1* mRNA accumulated in the endosperm that surrounded the developing embryo from the globular to the walking-stick stage and since a defect in cuticle formation was observed at these stages, it seems likely that ALE1 might function at the embryo's periphery to promote cuticle formation. In *A. thaliana*, the endosperm is a transient structure derived from double fertilization and it is thought to function as a source of nutrients that support the growth and development of the embryo. Appropriate development of the endosperm is a prerequisite for embryogenesis (Kinoshita et al., 1999). The expression pattern of *ALE1* suggests that the endosperm might also play a role in formation of the embryo's surface, in addition to its role in supplying nutrients to the embryo. ALE1 might also function within the embryo to integrate its shape since *ALE1* mRNA was also detected in the globular-stage embryo and the morphology of the developing embryo is affected in *ale1* seeds. Even though no *ALE1* RNA was detected in embryos after the heart stage, we cannot rule out the possibility that the *ALE1* gene, expressed in the embryo at a low levels beneath the limits of detection, might also be required for cuticle formation.

We only detected *ALE1* mRNA during seed development and this observation is consistent with the observation that *ale1* plants rarely show defects in the adult phase, even though plants have morphological defects in their cotyledons and early leaves (Fig. 1L). In addition, the cuticle was discontinuous on the surface of *ale1* cotyledons after germination. The surface areas of wild-type cotyledons in dry seeds increase by about 50-fold during 6 days after sowing (Tsukaya et al., 1994). Therefore, wild-type cotyledons of seedlings must produce cuticle de novo to cover their surface continuously. Our results suggest that the capacity for cuticle formation, acquired during seed formation, might be required for formation of the continuous cuticle that covers the rapidly growing seedling and allows its appropriate development.

A possible role of ALE1 in epidermal differentiation in *Arabidopsis*

The *ALE1* gene encodes a protein that shows similarity to members of subtilisin-like serine protease family. Several eukaryotic members of this family of proteases are involved in intercellular signaling, converting their substrates to either an active or an inactive form (Julius et al., 1984; Rose et al., 1996; Cui et al., 1998). For example, the yeast protease KEX2 cleaves and activates two peptides, killer toxin and α-factor (Julius et al., 1984); furin and/or PC6 in *Xenopus* activate BMP4 by proteolysis (Cui et al., 1998); and human tripeptidyl peptidase II inactivates a neurotransmitter, cholecystokinin (Rose et al., 1996). A plant member of this family, p69, is involved in the processing of a protein with leucine-rich repeats, suggesting a similar mode of action (Tornero et al., 1996b). The *SDD1* gene of *Arabidopsis*, which encodes another member of this family, is required for the control of cell lineage that leads to formation of stomatal guard cells (Berger and Altmann, 2000) but the biochemical activity and substrate of the gene product remain to be elucidated. Moreover, like many other members of this family of proteases, the predicted amino acid sequence of ALE1 includes a putative signal sequence for secretion (von Heijne, 1986) at the amino terminus (Fig. 4B). Lacking any known retention signal (Nakai and Kanehisa, 1992), ALE1 might be secreted from cells and function at the surface of the embryo. By analogy to these examples, ALE1 may also function in the production of a peptide ligand, which is required for proper differentiation of epidermis. The recent study of maize *crinkly4* (*cr4*) has also implicated the presence of signaling molecules that regulate epidermal differentiation of higher plants. *cr4* mutants of maize are defective in several epidermal characteristics, having irregular cell morphology and discontinuous cuticle, with the graft-like fusion of lateral organs (Becraft et al., 1996; Jin et al., 2000). The *CR4* gene appears to encode a receptor protein kinase whose extracellular

domain contains a cysteine-rich region that is similar to the ligand-binding domain in mammalian receptors for tumor necrosis factor. Thus, it is possible that differentiation of the plant epidermis might involve a peptide ligand (Becraft et al., 1996). In vertebrates, various peptide ligands, such as BMP4, BMP2 and BMP7, are involved in induction of epidermis formation. BMP4 is proteolytically activated by member(s) of the subtilisin-related proprotein convertase family in *Xenopus* embryos (Graff, 1997; Cui et al., 1998). In *C. elegans*, another subtilisin-like serine protease, BLI-4, is involved in cuticle formation (Thacker et al., 1995). It appears that subtilisin-like serine proteases are involved in epidermal differentiation in plants and animals, but this might be a coincidence as there is wide application of these proteases in both kingdoms. It, however, will be of interest to determine whether members of this family of protease are universally involved in epidermal differentiation in multicellular organisms.

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REFERENCES

Abe, M., Takahashi, T. and Komeda, Y. (1999). Cloning and characterization of an L1 layer-specific gene in *Arabidopsis thaliana*. *Plant Cell Physiol.* **40**, 571-580.

Baker, E. A. and Martin, J. T. (1963). Cutin of plant cuticles. *Nature* **28**, 1268-1270.

Batchelor, A. K., Boutilier, K., Miller, S. S., Labbe, H., Bowman, L., Hu, M., Johnson, D. A., Gijzen, M. and Miki, B. L. (2000). The seed coat-specific expression of a subtilisin-like gene, *SCS1*, from soybean. *Planta* **211**, 484-492.

Becraft, P. W., Stinard, P. S. and McCarty, D. R. (1996). CRINKLY4: A TNFR-like receptor kinase involved in maize epidermal differentiation. *Science* **273**, 1406-1409.

Berger, D. and Altmann, T. (2000). A subtilisin-like serine protease involved in the regulation of stomatal density and distribution in *Arabidopsis thaliana*. *Genes Dev.* **14**, 1119-1131.

Bogacheva, A. M., Rudenskaya, G. N., Preusser, A., Tchikileva, I. O., Dunaevsky, Y. E., Golovkin, B. N. and Stepanov, V. M. (1999). A new subtilisin-like proteinase from roots of the dandelion *Taraxacum officinale* Webb S. L. *Biochemistry* **64**, 1030-1037.

Bowman, J. L. and Mansfield, S. G. (1993). Embryogenesis. In *Arabidopsis: An Atlas of Morphology and Development* (ed. J. L. Bowman), pp. 350-401: Springer-Verlag.

Brongniart, A. (1834). Nouvelles recherches sur la structure de l'epiderme des vegetaux. *Ann. Sci. Nat*. **1**, 65-71.

Bruck, D. K. and Walker, D. B. (1985). Cell determination during embryogenesis in *Citrus jambhiri*. I. Ontogeny of the epidermis. *Bot. Gaz.* **146**, 188-195.

Cui, Y., Jean, F., Thomas, G. and Christian, J. L. (1998). BMP-4 is proteolytically activated by furin and/or PC6 during vertebrate embryonic development. *EMBO J.* **17**, 4735-4743.

Dure III, L. S. (1975). Seed formation. *Annu. Rev. Plant Physiol*. **26**, 259-278.

Goldberg, R. B., de Paiva, G. and Yadegari, R. (1994). Plant embryogenesis: Zygote to seed. *Science* **266**, 605-614.

Graff, J. M. (1997). Embryonic patterning: to BMP or not to BMP, that is the question. *Cell* **89**, 171-174.

Hamada, S., Onouchi, H., Tanaka, H., Kudo, M., Liu, Y. G., Shibata, D., Machida, C. and Machida, Y. (2000). Mutations in the *WUSCHEL* gene of *Arabidopsis thaliana* result in the development of shoots without juvenile leaves. *Plant J.* **24**, 91-101.

Holloway, P. J. (1982). The chemical constitution of plant cutins. In *The Plant Cuticle* (ed. D. F. Cutler, K. L. Alvin and C. E. Price), pp. 45-85. London: Academic Press.

Howell, S. H. (1998). *Molecular Genetics of Plant Development.* pp. 83. Cambridge: Cambridge University Press.

Jin, P., Guo, T. and Becraft, P. W. (2000). The maize CR4 receptor-like kinase mediates a growth factor-like differentiation response. *Genesis* **27**, 104-116.

Johnstone, I. L. (2000). Cuticle collagen genes. Expression in *Caenorhabditis elegans. Trends Genet.* **16**, 21-27.

Jorda, L., Coego, A., Conejero, V. and Vera, P. (1999). A genomic cluster containing four differentially regulated subtilisin-like processing protease genes is in tomato plants. *J. Biol. Chem.* **274**, 2360-2365.

Jorda, L., Conejero, V. and Vera, P. (2000). Characterization of P69E and P69F, two differentially regulated genes encoding new members of the subtilisin-like proteinase family from tomato plants. *Plant Physiol.* **122**, 67-74.

Julius, D., Brake, A., Blair, L., Kunisawa, R. and Thorner, J. (1984). Isolation of the putative structural gene for the lysine-arginine-cleaving endopeptidase required for processing of yeast prepro-alpha-factor. *Cell* **37**, 1075-1089.

Kaneda, M. and Tominaga, N. (1975). Isolation and characterization of a proteinase from the sarcocarp of melon fruit. *J. Biochem*. **78**, 1287-1296.

Kaneda, M., Ohmine, H., Yonezawa, H. and Tominaga, N. (1984). Amino acid sequence around the reactive serine of cucumisin from melon fruit. *J. Biochem. (Tokyo)* **95**, 825-829.

Kinoshita, T., Yadegari, R., Harada, J. J., Goldberg, R. B. and Fischer, R. L. (1999). Imprinting of the *MEDEA* polycomb gene in the *Arabidopsis* endosperm. *Plant Cell* **11**, 1945-1952.

Kobayashi, T., Kobayashi, E., Sato, S., Hotta, Y., Miyajima, N., Tanaka, A. and Tabata, S. (1994). Characterization of cDNAs induced in meiotic prophase in lily microsporocytes. *DNA Res*. **1**, 15-26.

Koizumi, K., Sugiyama, M. and Fukuda, H. (2000). A series of novel mutants of *Arabidopsis thaliana* that are defective in the formation of continuous vascular network: calling the auxin signal flow canalization hypothesis into question. *Development* **127**, 3197-3204.

Kolattukudy, P. E. (1970). Biosynthesis of cuticular lipids. *Annu. Rev. Plant Physiol.* **21**, 163-192.

Kolattukudy, P. E. (1980a). Cutin, Suberin, and Waxes. In *The Biochemistry of Plants*, vol. 4 (ed. P. K. Stumpf), chap. 18: Academic Press.

Kolattukudy, P. E. (1980b). Biopolyester membranes of plants: Cutin and suberin. *Science* **208**, 990-1000.

Laplaze, L., Ribeiro, A., Franche, C., Duhoux, E., Auguy, F., Bogusz, D. and Pawlowski, K. (2000). Characterization of a *Casuarina glauca* nodule-specific subtilisin-like protease gene, a homolog of *Alnus glutinosa ag12*. *Mol. Plant-Microbe Interact.* **13**, 113-117.

Lolle, S. J., Cheung, A. Y. and Sussex, I. M. (1992). *Fiddlehead*: an *Arabidopsis* mutant constitutively expressing an organ fusion program that involves interactions between epidermal cells. *Dev. Biol.* **152**, 383-392.

Lolle, S. J., Hsu, W. and Pruitt, R. E. (1998). Genetic analysis of organ fusion in *Arabidopsis thaliana*. *Genetics* **149**, 607-619.

Lolle, S. J. and Pruitt, R. E. (1999). Epidermal cell interactions: a case for local talk. *Trends Plant Sci.* **4**, 14-20.

Long, J. A. and Barton, M. K. (1998). The development of apical embryonic pattern in *Arabidopsis*. *Development* **125**, 3027-3035.

Lu, P., Porat, R., Nadeau, J. A. and O'Neill, S. D. (1996). Identification of a meristem L1 layer-specific gene in *Arabidopsis* that is expressed during embryonic pattern formation and defines a new class of homeobox genes. *Plant Cell* **8**, 2155-2168.

Machida, C., Onouchi, H., Koizumi, J., Hamada, S., Semiarti, E., Torikai, S. and Machida, Y. (1997). Characterization of the transposition pattern of the *Ac* element in *Arabidopsis thaliana* using endonuclease I-SceI. *Proc. Natl. Acad. Sci. USA* **94**, 8675-8680.

Martin, J. T. and Juniper, B. E. (1970). *The Cuticle of Plants*, pp. 1-11. London: Edward Arnold.

Meichtry, J., Amrhein, N. and Schaller, A. (1999). Characterization of the subtilase gene family in tomato (*Lycopersicon esculentum* Mill.). *Plant Mol. Biol.* **39**, 749-760.

Millar, A. A., Clemens, S., Zachgo, S., Giblin, E. M., Taylor, D. C. and Kunst, L. (1999). *CUT1*, an *Arabidopsis* gene required for cuticular wax biosynthesis and pollen fertility, encodes a very-long-chain fatty acid condensing enzyme. *Plant Cell* **11**, 825-838.

Nakai, K. and Kanehisa, M. (1992). A knowledge base for predicting protein localization sites in eukaryotic cells. *Genomics* **14**, 897-911.

Neuteboom, L. W., Veth-Tello, L. M., Clijdesdale, O. R., Hooykaas, P. J. and van der Zaal, B. J. (1999). A novel subtilisin-like protease gene from Arabidopsis thaliana is expressed at sites of lateral root emergence. *DNA Res.* **6**, 13-19.

Nishimura, M., Takeuchi, Y., De Bellis, L. and Hara-Nishimura, I. (1993). Leaf peroxisomes are directly transformed to glyoxysomes during senescence of pumpkin cotyledons. *Protoplasma* **175**, 131-137.

Pruitt, R. E., Vielle-Calzada, J. P., Ploense, S. E., Grossniklaus, U. and Lolle, S. J. (2000). *FIDDLEHEAD*, a gene required to suppress epidermal cell interactions in Arabidopsis, encodes a putative lipid biosynthetic enzyme. *Proc. Natl. Acad. Sci. USA* **97**, 1311-1316.

Ribeiro, A., Akkermans, A. D., van Kammen, A., Bisseling, T. and Pawlowski, K. (1995). A nodule-specific gene encoding a subtilisin-like protease is expressed in early stages of actinorhizal nodule development. *Plant Cell* **7**, 785-794.

Rodkiewicz, B., Fyk, B. and Szczuka, E. (1994). Chlorophyll and cutin in early embryogenesis in *Capsella*, *Arabidopsis*, and *Stellaria* investigated by fluorescence microscopy. *Sex. Plant Reprod.* **7**, 287-289.

Rose, C., Vargas, F., Facchinetti, P., Bourgeat, P., Bambal, R. B., Bishop, P. B., Chan, S. M., Moore, A. N., Ganellin, C. R. and Schwartz, J. C. (1996). Characterization and inhibition of a cholecystokinin-inactivating serine peptidase. *Nature* **380**, 403-409.

Rudenskaya, G. N., Bogdanova, E. A., Revina, L. P., Golovkin, B. N. and Stepanov, V. M. (1995). Macluralisin – a serine proteinase from fruits *of Maclura pomifera* (Raf.) Schneid. *Planta* **196**, 174-179.

Rudenskaya, G. N., Bogacheva, A. M., Preusser, A., Kuznetsova, A. V., Dunaevsky Ya, E., Golovkin, B. N. and Stepanov, V. M. (1998). Taraxalisin – a serine proteinase from dandelion *Taraxacum officinale* Webb s.l. *FEBS Lett.* **437**, 237-240.

Semiarti, E., Ueno, Y., Tsukaya, H., Iwakawa, H., Machida, C. and Machida, Y. (2001). The *ASYMMETRIC LEAVES2* gene of *Arabidopsis thaliana* regulates formation of a symmetric lamina, establishment of venation and repression of meristem-related homeobox genes in leaves. *Development* **128**, 1771-1783.

Sieber, P., Schorderet, M., Ryser, U., Buchala, A., Kolattukudy, P., Metraux, J. P. and Nawrath, C. (2000). Transgenic *Arabidopsis* plants expressing a fungal cutinase show alterations in the structure and properties of the cuticle and postgenital organ fusions. *Plant Cell* **12**, 721-738.

Sinha, N. and Lynch, M. (1998). Fused organs in the *adherent1* mutant in maize show altered epidermal wall with no perturbations in tissue identities. *Planta* **206**, 184-195.

Sterk, P., Booij, H., Schellekens, G. A., Van Kammen, A. and De Vries, S. C. (1991). Cell-specific expression of the carrot EP2 lipid transfer protein gene. *Plant Cell* **3**, 907-921.

Thacker, C., Peters, K., Srayko, M. and Rose, A. M. (1995). The *bli-4* locus of Caenorhabditis elegans encodes structurally distinct kex2/subtilisin-like endoproteases essential for early development and adult morphology. *Genes Dev.* **9**, 956-971.

Tornero, P., Conejero, V. and Vera, P. (1996a). Primary structure and expression of a pathogen-induced protease (PR-P69) in tomato plants: Similarity of functional domains to subtilisin-like endoproteases. *Proc. Natl. Acad. Sci. USA* **93**, 6332-6337.

Tornero, P., Mayda, E., Gomez, M. D., Canas, L., Conejero, V. and Vera, P. (1996b). Characterization of LRP, a leucine-rich repeat (LRR) protein from tomato plants that is processed during pathogenesis. *Plant J.* **10**, 315-330.

Tornero, P., Conejero, V. and Vera, P. (1997). Identification of a new pathogen-induced member of the subtilisin-like processing protease family from plants. *J. Biol. Chem.* **272**, 14412-14419.

Tsukaya, H., Tsuge, T. and Uchimiya, H. (1994). The cotyledon: A superior system for studies of leaf development. *Planta* **195**, 309-312.

Van Lammeren, A. A. M. (1986). Developmental morphology and cytology of the young maize embryo (*Zea Maize* L.). *Acta Bot. Neerl.* **35**, 169-188.

Vera, P. and Conejero, V. (1988). Pathogenesis-related proteins of tomato. P69 as an alkaline endoproteinase. *Plant Physiol.* **87**, 58-63.

von Heijne, G. (1986). A new mothod for predicting signal sequence cleavage sites. *Nucleic Acids Res.* **14**, 4683-4690.

Yamagata, H., Masuzawa, T., Nagaoka, Y., Ohnishi, T. and Iwasaki, T. (1994). Cucumisin, a serine protease from melon fruits, shares structural homology with subtilisin and is generated from a large precursor. *J. Biol. Chem.* **269**, 32725-32731.

Yamagata, H., Uesugi, M., Saka, K., Iwasaki, T. and Aizono, Y. (2000). Molecular cloning and characterization of a cDNA and a gene for subtilisin-like serine proteases from rice (*Oryza sativa* L.) and *Arabidopsis thaliana*. *Biosmci. Biotechnol. Biochem.* **64**, 1947-1957.

Yephremov, A., Wisman, E., Huijser, P., Huijser, C., Wellesen, K. and Saedler, H. (1999). Characterization of the *FIDDLEHEAD* gene of *Arabidopsis* reveals a link between adhesion response and cell differentiation in the epidermis. *Plant Cell* **11**, 2187-2201.