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 shape (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 crf4 mutants show irregularities in the structure of cell walls and the formation of cuticle (Jin et al., 2000). The CRF4 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 CRF4 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; Jordà et al., 1999; Meichtry et al., 1999; Neuteboom et al., 1999; Batchelor et al., 2000; Berger and Altmann, 2000; Jordà 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, 2000; Yamagata et al., 2000) and some of them were shown to have protease activities in vitro (Kaneda and Tominaga, 2000; Yamagata et al., 2000).

Materials and methods

Plants and growth conditions

Arabidopsis thaliana ecotype Landsberg erecta (Ler) 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 M2 population of Ler 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'-CTAATGGAACTTAAATCCAAG-3') and ALE1-3'U3 (5'-TTTCTCAATGGTTTTAATCTGAAAC-3'). For rapid amplification of cDNA ends (RACE), first-strand cDNA was synthesized with the primer ALE1RTP (5'-CTGGCTTTACCTCTTT-3') and self-ligated. RACE was performed with primers A1_5'S1 (5'-GGGAAGCACAATCTGGAGGGA-3') and A1_5'A1 (5'-GCTC-TGGACAATATGGCACA-3'), and nested PCR was performed with primers A1_5'S2 (5'-CTTTTGGAGCTGGTACCA-3') and A1_5'A2 (5'-CATATCATGGCGATGGACA-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'S1 (5'-CGCCACAGGATCTTGTTGACACTAGCAGCACACCC-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.,
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 BamHI-XhoI fragment from pALE13RU was used to generate a hybridization probe. In situ hybridization was performed as described (Long and Barton, 1998; Semiatari 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 BamHI and synthesized using T7 RNA polymerase. Sense control probes were synthesized using T7 RNA polymerase on pALE115146 linearized with BamHI. 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 M2 population of EMS-mutagenized Ler 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.
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 micropyllar 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. 2J), 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).
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 alel-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 alel-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 alel-1 mutation among 80 F2 plants.

As shown in Fig. 3, four independent revertants (alel-1R1 through R4) were obtained from approx. 3,000 alel 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 alel-1 gene. Southern hybridization using the genomic fragment as probe also

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

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. The relative positions of the alel 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 (the catalytic triad) are indicated (see B,C). The relative positions of the alel 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 (the catalytic triad) are indicated (see B,C). The relative positions of the alel 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 (the catalytic triad) are indicated (see B,C). The relative positions of the alel mutations are shown.

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.
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. 4A,B).

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 (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.

**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.
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 (fhd) 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 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 (Kinosita 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 (Torniero 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 Altman, 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.

We thank Dr Robert L. Fischer (University of California, Berkeley) for critical reading of the manuscript; Dr Hidemi Kitano (Dept. of Agriculture, Nagoya University) for helpful discussions and suggestions related to histological analysis; Prof. Fumio Osawa (Aichi Institute of Technology) for his encouragement and support; Dr Ryoichi Nishihama (Dept. of Biology, University of North Carolina) for helpful discussion and suggestions related to expression analyses. The authors also thank Mr. Takaaki Ishikawa (Div. of Biological Science, Nagoya University) for providing the transformation vector pGAB2, and Yasutaka Katsumata for technical assistance. This work was supported in part by grants from the Research for the Future Program of the Japan Society for the Promotion of Science (JSPS-RFTF97L00601 and JSPS-RFTF00L01663), by Grants-in-Aid for Scientific Research on Priority Areas (no. 10182101) and for General Scientific Research Projects in Biotechnology from the Ministry of Agriculture, Forestry and Fisheries of Japan, H. T. Is a JSPS Research Fellow. C. M. was supported by a grant from the Central Laboratories for Key Technology, Kirin Brewery Co., Ltd.

REFERENCES


