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Cloning of Epidermis-specific cDNAs Encoding a Lipid Transfer Protein and an Aldehyde Decarbonylase from *Senecio odorus*

Pyee, Jaeho

Department of Molecular Biology, Dankook University, Seoul 140-714, Korea

The major cuticular components have been shown to be synthesized in the epidermis. Therefore, cloning of epidermis-specific genes could yield information to be used to isolate and characterize the enzymes involved in the cuticle biosynthesis. A subtractive cDNA library was prepared from *Senecio odorus* in which epidermis-specific cDNAs were enriched. Differential screening of the library using epidermal and non-epidermal probes revealed two cDNAs. One of them designated *epi425* was identified, based on the sequence homology, as a member of a new class in the LTP gene family and the other clone designated *epi23* as a gene encoding an aldehyde decarbonylase. Northern blot analyses showed that *epi425* and *epi23* cDNAs hybridized with a transcript of about 600 and 2,100 nucleotides, respectively, from the epidermis but not from the non-epidermal tissues. Further characterization of these clones will provide more information on the mechanism of the cuticle biosynthesis.

Keywords : epidermis, cDNA, lipid transfer protein, aldehyde decarbonylase, *Senecio odorus*

The epidermal tissue, being the outermost layer of cells helps to protect the underlying tissues (mesophyll and vascular tissues) from physical, chemical and biological stresses such as dehydration, pathogens, UV etc. The epidermis of the aerial parts of plants are covered with the cuticle; physical and chemical characteristics of the cuticle contribute to many of the properties of the epidermis (Martin and Juniper, 1970; Kolattukudy, 1987). The cuticle consists of an insoluble biopolyester, cutin, embedded in a complex mixture of highly hydrophobic materials collectively called wax. These cuticular components have been shown to be synthesized in the epidermis and secreted to the surface (Kolattukudy, 1968; Croteau and Kolattukudy, 1974; Cheesbrough and Kolattukudy, 1984). Although the basic biochemical pathways involved in the biosynthesis of all of the major cuticular components have been elucidated and the enzymes postulated to be involved in the processes have been demonstrated in cell-free preparations (Croteau and Kolattukudy, 1973; Khan and Kolattukudy, 1974; Croteau and Kolattukudy, 1974; Croteau and Kolttukudy, 1975; Cheesbrough and Kolattukudy, 1984), only a few of them have been isolated and characterized. Isolation and characterization of epidermis-specific genes could yield information that could lead to the isolation and characterization of the enzymes involved in the cuticle biosynthesis.

Senecio odorus (*Kleinia odora*) has been used as a convenient system for isolating epidermis (Kuiper, 1964; Kolattukudy, 1968), since this plant allowed to easily prepare the epidermis with little contamination from the non-epidermal tissues. Such excised epidermal tissues have been used to elucidate the enzymology of the biosynthesis of the cuticular components (Kolattukudy, 1968; Croteau and Kolattukudy, 1974; Kolattukudy, 1980).

Several genes have been reported to be specifically expressed in the epdermis of various plants. Each gene showed a different pattern of epidermal expression. For example, expression of the chalcone synthase and phenylalanine ammonia-lyase gene families were induced in the epidermis by UV or infection by pathogens (Schmelzer *et al.*, 1988; Schmelzer *et al.*, 1989). A tobacco lipid transfer protein (LTP) transcript was localized by *in situ* hy-

Corresponding author: Fax +82-2-793-0176

bridization in the epidermis of leaf but in stem sections epidermis-specific expression was not seen (Fleming *et al.*, 1992). Some of the epidermis-specific genes were found to be expressed in the epidermis of specific organs such as petals (Drews *et al.*, 1992) and roots (Pichon *et al.*, 1992). Recently, several more epidermis-specific cDNAs were isolated by differential screening of a cDNA library prepared from the epidermis of *Pachyphytum* (Clark *et al.*, 1992). The transcripts corresponding to these cDNAs were expressed constitutively, abundantly and exclusively in the epidermis and one of the cDNAs (EPI12) was found to encode a typical LTP (Clark and Bohnert, 1993).

I prepared a subtractive cDNA library from *Senecio odorus* in which epidermis-specific cDNAs were enriched after subtractive hybridization with non-epidermal cDNAs and screened the library to search for genes involved in the epidermis-specific functions. Two cDNAs representing such genes were isolated. One of them was identified, based on the sequence homology, as a member of a new class in the LTP gene family. The other clone showed a high sequence homology to a cDNA encoding decarbonylase isolated from *Arabidopsis*.

MATERIALS AND METHODS

Plant materials

Young leaves of *Senecio odorus* (Forssk) Defl. (or *Kleinia odora* (Forssk) DG.) grown in a green house were taken and both the upper and lower epidermal layers were peeled off (Kolattukudy, 1968). The epidermal layers and the non-epidermal (underlying) tissues containing mesophyll and vascular tissues were separately frozen in liquid nitrogen and stored at $-70°C$. Stems, roots and whole leaves were also frozen.

RNA isolation

Total RNA was isolated as described by Jones *et al.* (1985). The epidermal and non-epidermal tissues (about 1 g) of leaves, stems and root tissues were separately ground in a mortar with liquid nitrogen to a fine powder. After adding 4.5 mL of NTES buffer (0.1 M NaCl, 0.01 M Tris-Cl, pH 7.5, 1 mM EDTA, 1% SDS) and 3 mL of a 25 : 24 : 1 mixture of phenol/chloroform/isoamyl alcohol, the mixture was ground until it thawed and the slurry was transferred to a centrifuge tube. After centrifugation at 10,000× g for 10 min, the supernatant was mixed with 0.1 volume of 3 M sodium acetate (pH 6.2) and 2 volume of ethanol. The resulting precipitate with an equal volume of 4 M lithium acetate on ice for at least 3 h. After centrifugation at 10,000× g for 10 min, the RNA pellet was dissolved in diethylpyrocarbonate-treated water and reprecipitated with 3 M sodium acetate and ethanol. Poly(A)$^+$ RNA was isolated from total RNA using an oligo-(dT) cellulose column as described (Sambrook *et al.*, 1989).

Construction and screening of a subtractive cDNA library

Poly(A)$^+$ RNAs (5 μg) extracted separately from the epidermis and the non-epidermal tissues were converted to double-stranded blunt-ended cDNAs using the Librarian cDNA Synthesis kit (Invitrogen). While the cDNA synthesized from the non-epidermal tissue was double-digested with AluI and RsaI, the cDNA synthesized from the epidermal tissue was ligated to EcoRI adaptors (Invitrogen). After denaturation, epidermal cDNA (0.1 μg) was hybridized with 50-fold excess of non-epidermal tissue cDNA in 50% formamide solution containing 5 x SSPE, 10 mM $NaPO_4$, pH 7.0, 1 mM EDTA, 0.1% SDS and 0.2 mg/mL yeast tRNA at 37°C for 18 h. The hybridization mixture was extracted with phenol/chloroform/isoamyl alcohol and precipitated with ethanol. Then, the pellet was dissolved in water and ligated to λgt 11 arms (Stratagene) so that the differentially expressed cDNAs with compatible EcoRI adaptors on both ends could be ligated to EcoRI-digested λgt 11 arms. The library was spread at a density of approximately 4-5,000 plaque forming units on plates containing ampicillin (50 μg/mL) and duplicate lifts made on nitrocellulose membranes were differentially screened with two different probes. The probes were the first strand cDNAs synthesized from the epidermal or the non-epidermal poly(A)$^+$ RNAs using reverse transcriptase (BRL) and α-^{32}P-dATP. Prehybridization and hybridization were carried out in 50% formamide, 5 x SSPE, 5 x Denhart's solution, 0.1% SDS and 100 μg/mL of denatured salmon sperm DNA at 42°C using standard procedures (Sambrook *et al.*, 1989). After hybridization, the blots were washed with 0.2 to 2.0 x SSPE and 0.1% SDS at room temperature to 65°C depending on the intensity of signals and exposed to X-ray films. Putative positive plaques which gave stronger signals with the epidermal probe were picked and analyzed. Phage DNA was isolated from the

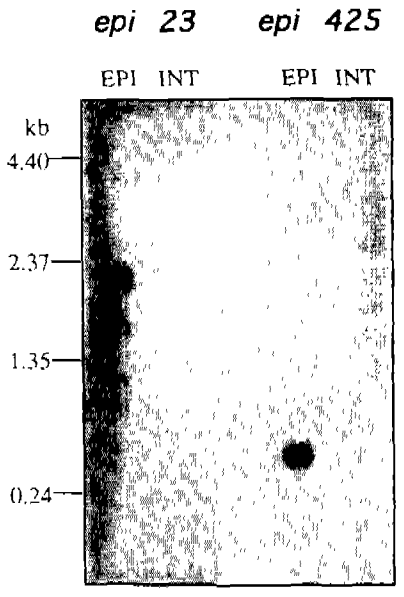

Fig. 1. Tissue-specific expression of *epi23* and *epi425* cDNAs. Total RNAs (10 μg) isolated from both epidermal and non-epidermal tissues were fractionated on a 1.2% agarose gel containing 0.66 M formaldehyde, blotted on a Nytran membrane (Schleicher & Schuell) and separately probed with the ^{32}P-labeled inserts of the putative cDNA clones (*epi23* and *epi425*). EPI, epidermal RNA; INT, internal (non-epidermal) RNA.

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1/1                                                 31/11
TCA GCG ATA TAC ATA GTA GCA GTT TTG GTG ATG ATC GTT GGC GGA TCC AAG GTG GCA ACA
S   A   I   Y   I   V   A   L   L   V   M   I   V   A   G   S   K   V   A   T

61/21                                               91/31
GCG GGG ACA TGC TCA GTG ACT GAG GTA ATG CCT TGT TCG TCG GCT TTT ACA TCA TCT GCT
A   A   T   C   S   V   T   E   L   M   P   C   S   S   A   F   T   S   S   A

121/41                                              151/51
GCA CCG ACG GCA CAA TGT TGT ACA AAG TTA AAA GAA CAG AGC CCA TGT GTA TGC GGG TAC
A   P   T   A   Q   C   C   T   K   L   K   E   Q   S   P   C   L   C   G   Y

181/61                                              211/71
TTA AAG AAC CCA ACC TTG AAG CAG TAT ATA ACT AAT CCT AAT GCA AAG AAG GTT ACT AGC
L   K   N   P   T   L   K   Q   Y   I   T   N   P   N   A   K   K   V   T   S

241/81                                              271
ACT TGT GGT GTT CCC ATT CCC AAT TGT TGA gtg ttt gaa tta ata gac cac ctc aca tgt
T   C   G   V   P   I   P   N   C   *

301                                                 331
taa aga aag tac tac tcc tat taa aca gtc gac gta cta tac agt att ctg tga tgt tct

361                                                 391
ttc act atc tca aga ttc ctg agt tgt caa gag atc ata tta tgt gcg gcg ccg aaa aaa

421                                                 451
aaa aaa aaa aaa aaa aaa aaa aaa aaa aaa aaa aaa aaa aaa aaa aaa aaa aaa aaa aaa

481
aaa aaa aaa aaa
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Fig. 2. The nucleotide and the amino acid sequences of the *epi425* cDNA. Both strands of the *epi425* cDNA were sequenced using Sequenase (USB) by the deoxy termination method. The largest open reading frame found using the DNA Strider software encodes a polypeptide of 89 amino acids (capical letters) missing the putative N-terminal end. All of the amino acid residues are shown in the one-letter code. Methionine residues are in bold-face.

of 9.7. The deduced protein has 8 cysteine residues. According to the hydropathy plot (Kyte and Doolittle, 1982), the deduced protein has a hydrophobic region of 40 amino acids in the N-terminal part of which the first hydrophobic domain resembles the putative leader peptides of other homologous proteins (data not shown). Thus, the *epi425* open reading frame appears to have a part of the region encoding the putative leader peptide and the following region corresponding to the processed mature polypeptide.

```
                1                                                   50
EPI425                $ AIYIVALLVM IV....AGSK VATAATCSVT ELMPCSSAFT
p48h10          MRSITYT SLYAAAILMM IL....AGSQ TTMAVTCLVT QLAPCASAIS
B11E         MAMAMGMAMR KEAAVAVMMV MVVTLAAGAD AGAGAACEPA QLAVCASAIL
PAP058             MV KVMWVSVLAL AAAILLLTVP VAEGVTCSPM QLASCLAAMT

                51                                                  100
EPI425       SSAAPTAQCC TKLKEQSPCL CGYLKNPTLK QYITNPNAKK VTSTCGVPIP
p48h10       SSSPPSSQCC AKIKEQKPCL CQYMKNPSLK AYVSSPNAKK VANACGVPIP
B11E         GGTKPSGECC GNLRAQQGCL CQYVKDPNYG HYVSSPHARD TLNLCGIPVP
PAP058       SSSPQSEACC TKLREQQPCL CGYMRNPTLR QYVSSPNARK VSNSCKIPSP

                101
EPI425       NC
p48h10       KC
B11E         HC
PAP058       SC
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Fig. 3. Similarity among EPI425, P48h-10, B11E and PAP058. Comparison among the amino acid sequences of the deduced polypeptides from the putative open reading frames of these clones were performed using the GCG software. The completely conserved Cysteine residues are underlined. Gaps(.) are added for the best alignment.

The predicted protein shares high similarity (31 to 59% identity and 71 to 81% similarity) to a 10-kDa protein deduced from a barley aleurone-specific cDNA B11E (Jacobsen *et al.*, 1989), an *Arabidopsis* polypeptide deduced from a seed cDNA PAP058 (Z 27019 in GenBank) and a protein deduced from a recently isolated *Zinnia* cDNA, p48h-10, associated with tracheary element formation (Varner, 1993) (Fig. 3). The deduced proteins all have 8 cysteine residues at identical positions. The hydropathy plots of these proteins showed a high degree of similarity as expected from the sequence similarity and they probably have similar structures.

The B11 clone was found to represent a gene exclusively expressed in aleurone and was proposed to encode a lipid transfer protein (LTP). PAP058 cDNA clone, isolated from dry seeds of *Arabidopsis*, also revealed an open reading frame encoding a lipid transfer protein. However, the pattern of spacing between the conserved cysteine residues of these proteins is slightly different from the typical pattern of spacings found in other plant LTPs (data not

putative positives using the standard procedures (Sambrook, *et al.*, 1989) and the inserts were subcloned into pBluescript II KS(+) vector (Stratagene) for further analysis.

Northern blot analysis

Poly(A)$^+$ RNAs or total RNAs prepared separately from the epidermis and the non-epidermal (internal) tissues of leaves and stems were fractionated on 1. 2% agarose gel containing 0.66 M formaldehyde, blotted on Nytran membranes (Schleicher & Schuell) in 10 x SSPE and hybridized with the ^{32}P-labeled inserts of the putative clones. Prehybridization, hybridization and washing were carried out as described above.

DNA sequencing

Both strands of the inserts of the positive clones were sequenced using Sequenase kit (Version 2.0, United State Biochemicals) by the dideoxy termination method (Sanger *et al.*, 1977). Analysis of the nucleotide and the deduced amino acid sequences were performed at the NCBI using the BLAST network service.

RESULTS AND DISCUSSION

Construction and screening of a subtractive cDNA library

One of the unique functions of the epidermal layer of cells is to produce the cuticle covering the plant organ. Epidermal cells have been shown to be the site for biosynthesis of the cuticular components (Kolattukudy, 1968; Croteau and Kolattukudy, 1974; Cheesbrough and Kolattukudy, 1984; Kolattukudy, 1987) and epidermal tissues have been used to prepare cell-free preparations capable of catalyzing reactions uniquely involved in the cuticle biosynthesis (Croteau and Kolattukudy, 1973; Croteau and Kolattukudy, 1974; Khan and Kolattukudy, 1974; Croteau and Kolattukudy, 1975; Cheesebrough and Kolattukudy, 1984). With the technical difficulties in isolating epidermal tissues in amounts adequate for conventional protein fractionation procedures the enzymes involved in the cuticle biosynthesis have not been purified and characterized yet. An alternative way to elucidate the nature of such epidermis-specific enzymes is to isolate and characterize corresponding genes and use such information to study the enzymes. Some of the isolated genes should be related to cuticle biosynthesis. In order to study genes that are uniquely expressed in the epidermis, I constructed a subtractive cDNA library enriched for epidermal cDNA. *Senecio odorus* has been used to prepare the epidermis with little contamination from the non-epidermal tissues and such excised epidermal tissues have been previously used to elucidate the enzymology of biosynthesis of the cuticular components (Kolattukudy, 1968; Croteau and Kolattukudy, 1974; Kolattukudy, 1980).

Epidermal cDNA was subtracted with 50-fold molar excess of double restriction enzyme-digested non-epidermal cDNA and the enriched epidermis-specific cDNA was cloned in λgt 11. The titer of the unamplified library was 6.6×10^4 plaque forming units (pfu). In order to obtain well isolated individual plaques in the first screening, the library was plated at a low density (4-5,000 pfu).

Differential screening of a portion of the library provided 2 putative positives, designated *epi23* and *epi425*, which hybridized more strongly or exclusively with the epidermal probe. Differential expression of the putative clones was further confirmed by northern blot analysis.

Epidermis-specific expression of the differentially-regulated transcripts

Northern blot analysis showed that *epi23* and *epi 425* cDNA hybridized with a transcript of about 2, 100 and 600 nucleotides, respectively, from the epidermis but not from the non-epidermal tissues (Fig. 1). *epi425* was abundantly expressed in the epidermis of leaves and stems but very low level expression could also be detected in the non-epidermal tissues of leaves and stems by a 31 h exposure of the blot to film (data not shown). The other clone, *epi23* was expressed exclusively in the epidermis of leaves. However, its expression in other tissues such as stems and roots needs to be tested further.

Sequence analysis of the *epi425* clone

The nucleotide and the deduced amino acid sequences of the *epi425* cDNA were obtained and analyzed (Fig. 2). The cDNA did not represent the full length transcript of about 600 nucleotides as indicated by northern blot analysis. It contains an incomplete open reading frame (ORF) encoding a polypeptide of 89 amino acids with an estimated molecular mass of 9.2 kDa and an isoelectric point

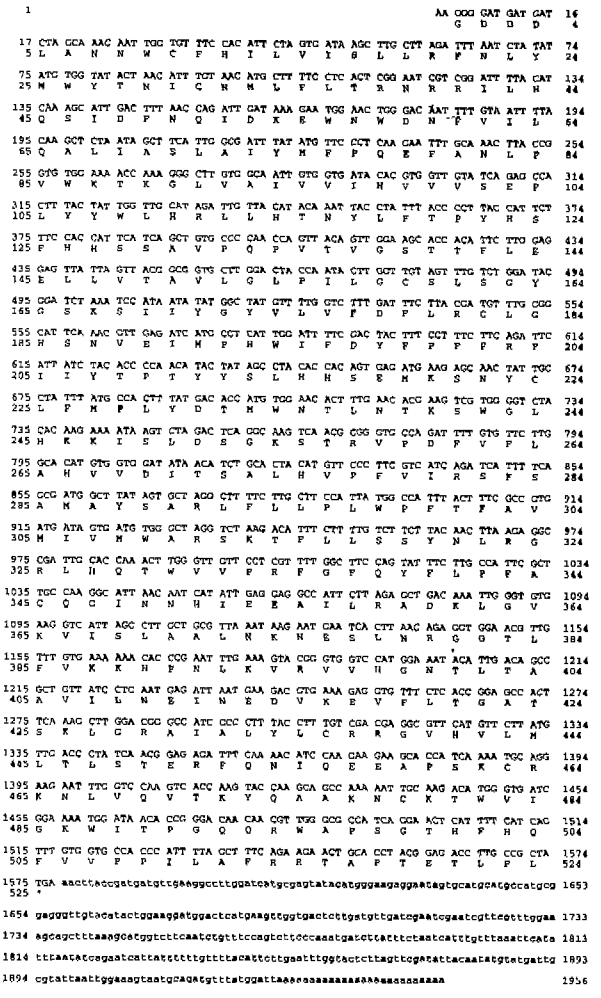

Fig. 4. The nucleotide and amino acid sequences of the *epi23* cDNA. Both strands of the *epi23* cDNA were sequenced using Sequenase (USB) by the deoxy termination method. The largest open reading frame found using the DNA Strider software encodes a polypeptide of 524 amino acids (capical letters) missing the putative N-terminus. All of the amino acid residues are shown in the one-letter code.

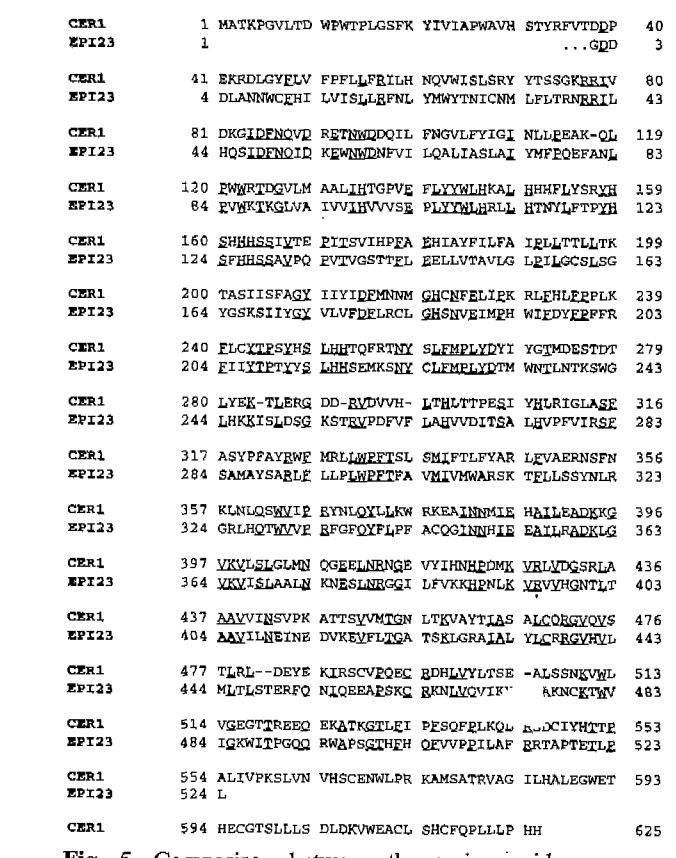

Fig. 5. Comparison between the amino acid sequences deduced from the putative open reading frames of *epi23* and *CER1* cDNAs. Identical amino acid residues are underlined. Gaps(-) are added for the best alignment.

shown), suggesting that these proteins may have a slightly different structure, constituting members of a new class in the LTP family. It remains to be determined whether this structural similarity among the four deduced proteins including EPI425 suggests a functional similarity among them in the different anatomical regions where they were isolated.

Sequence analysis of the *epi23* clone

The isolated *epi23* clone contains an open reading frame encoding a polypeptide of 524 amino acids (Fig. 4). It is not clear yet whether the ORF is complete. Although there are two methionin residues at 25th and 33th positions, the region immediately upstream of these methionine residues is still in-frame with the open reading frame. The cDNA lacked a consensus polyadenylation signal (AATAAA) but several other AT-rich regions were present that may serve this function. The predicted amino acid sequence showed 31.3% identity with the deduced amino acid sequence of the *CER1* gene recently isolated from an *Arabidopsis* mutant, *ECERIFERUM* (*CER*), which is blocked in the conversion of stem wax C30 aldehydes to C29 alkanes (Aarts *et al.*, 1995). This reaction is catalyzed by an aldehyde decarbonylase and aldehyde decarbonylases have been partially purified from algae and epidermal strips of various plants. However, *cer1* and *epi23* are the first cases to isolate the corresponding genes.

Analysis of the deduced amino acid sequence of *epi23* revealed that the deduced protein is predominantly hydrophobic especially at the N-terminus,

suggesting that this protein may be associated with the membrane. This is consistent with the finding that there are two putative membrane spanning sequences in the CER1 amino acid sequence. The membrane-associated localization of the decarbonylase has been demonstrated by the supporting experiments in other systems. For example, the decarbonylase activity in *Pisum sativum* was found to be associated with a particulate fraction containing cell-wall and cuticle fragmants (Kolattukudy, 1987). However, determination of the biosynthetic mechanism for hydrocarbons and characteristics of the involved enzyme needs further experiments on the corresponding gene and the purified or overexpressed protein from it.

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LITERATURE CITED

Aarts, M.G.M., C.J. Keijzer, W.J. Stiekema and A. Pereira. 1995. Molecular characterization of the *CER!* gene of *Arabidopsis* involved in epicuticular wax biosynthesis and pollen fertility. *Plant Cell* **7**: 2115-2127.

Cheesbrough, T.M. and P.E. Kolattukudy. 1984. Alkaline biosynthesis by decarbonylation of aldehydes catalyzed by a particulate preparation from *Pisum satium. Proc. Natl. Acad. Sci. USA* **81**: 6613-6617.

Clark, A.M. and H.J. Bohnert. 1993. Epidermis-specific transcripts. Nucleotide sequence of a full-length cDNA of EPI12, encoding a putative lipid transfer protein. *Plant Physiol.* **103**: 677-678.

Clark, A.M., J.A. Verbeke and H.J. Bohnert. 1992. Epidermis-specific gene expression in *Pachyphytum. Plant Cell* **4**: 1189-1198.

Croteau, R. and P.E. Kolattukudy. 1974. Biosynthesis of hydroxyfatty acid polymers. Enzymatic synthesis of cutin from monomer acids by cell-free preparations from the epidermis of *Vicia faba* leaves. *Biochemistry* **13**: 3193-3202.

Denecke, J., M.H.S. Goldman, J. Demolder, J. Seurinck and J. Botterrman. 1991. The tobacco luminal binding protein is encoded by a multigene family. *Plant Cell* **3**: 1025-1035.

Dennis, M.W. and P.E. Kolattukudy. 1991. Alkane biosynthesis by decarbonylation of aldehyde catalyzed by a microsomal preparation from *Botryococcus braunii. Arch. Biochem. Biophsys.* **287**: 268-275.

Drews, G.N., T.P. Beals, A.Q. Bui and R.B. Goldberg. 1992. Regional and cell-specific gene expression patterns during petal development. *Plant Cell* **4**: 1383-1404.

Fleming, A.J., T. Mandel, S. Hoffman, P. Sterk, S.C. de Vries and C. Kuhlemeier. 1992. Expression pattern of a tobacco lipid transfer gene within the shoot apex. *Plant J.* **2**: 855-862.

Fukuda, H. 1992. Trachearty element formation as a model system of cell differentiation. *International Review Cytology* **136**: 289-332.

Fukuda, H. and A. Komamine. 1985. Cytodifferentiation. *Cell culture and somatic cell genetics of plant* **2**: 149-212.

Jacobsen, J.V., R.B. Knox and N.A. Pyloits. 1971. The structure and composition of aleurone grains in the barley leurone layer. *Planta (Berl.)* **101**: 189-209.

Jones, J.D.G., P. Dunsmuir and J. Bedbrook. 1985. High level expression of introduced chimaeric genes in regenerated transformed plants. *EMBO J.* **4**: 2411-2418.

Kahn, A.A. and P.E. Kolattukudy. 1974. Decarboxylation of long chain fatty acids to alkanes by cell-free preparation of pea leaves (*Pisum sativum*). *Biochem. Biophys. Res. Comm.* **61**: 1379-1386.

Kolattukudy, P.E. 1968a. Biosynthesis of surface lipids. *Science.* **159**: 498-505.

Kolattukudy, P.E. 1968b. Further evidence for an elongation-decarboxylation mechanism in the biosynthesis of paraffins in leaves. *Plant Physiol.* **43**: 375-383.

Kolattukudy, P.E. 1980. Cutin, suberin and waxes. *In* The Biochemistry of Plants. vol. 4, PK, Stumpf (ed.), Academic Press, New York, pp. 571-645.

Kolattukudy, P.E. 1987. Lipid-derived defense polymers and waxes and their role in plant microbe interaction. *In* Lipids: structure and function. PK, Stumpf (ed.), Academic Press, Orlando, pp 291-314.

Kuniper, P.J.C. 1964. Dependence upon wavelength of stomatal movement in epidermal tissue in *Senecio odoris. Plant Physiol.* **39**: 952-955.

Kyte, J. and R.F. Doolittle. 1982. A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* **157**: 105-132.

Marbach, I. and A.M. Mayer. 1974. Permeability of seed coats to water as related to drying conditions and metabolism of phenolics. *Plant Physiol.* **54**: 817-820.

Martin, J.T. and B.E. Juniper. 1970. The cuticles of plants. St Marins Press, New York, pp. 147.

Molina, A., Segura, A. and Garcia-Olemedo. 1993. Lipid transfer proteins (nsLTP) from barkey and maize leaves are potent inhibitors of bacterial and fungal plant pathogens. *FEBS* **316**: 119-122.

Mundy, J. and J.C. Rogers. 1986. Selective expression of a probable amylase/proteae inhibitor in barley aleurone cells: Comparison to the barley amylase/subtilisin inhibitor. *Planta* **169**: 51-63.

Munro, S. and H.R.B. Pelham. 1987. An C-terminal sig-

nal prevents secretion of luminal ER proteins. *Cell* **48**: 899-907.

Sambrook, J., E.F. Fritsch and T. Maniatis. 1989. Molecular cloning: a laboratory manual. 2nd ed., Cold Spring Harbor Laboratory Press, New York.

Sanger, F., S. Nicklen, and A.R. Coulson. 1977. DNA sequencing with chain-terminating inhibition. *Proc. Natl. Acad. Sci. USA* **74**: 5463-5467.

Schmelzer, E., W. Jahnen and K. Hahlbrock. 1988. In situ localization of light-induced chalcone synthetase mRNA, chalcone synthetase, and flavonoid end products in epidermal cells of parsley leaves. *Proc. Natl. Acad. Sci. USA* **85**: 2989-2993.

Schmelzer, E., S.S. Kruger-Lebu and K. Hahlbrock. 1989. Temporal and spatial patterns of gene expression around sites of attempted fungal infection in parsley leaves. *Plant Cell* **1**: 993-1001.

Tsuboi, S., T. Suga, K. Takishima, G. Mamiya, K. Matsui, Y. Ozeki and M. Yamada. 1991. Organ-specific occurrence expression of the isoforms of nonspecific lipid transfer prtoein in castor bean seedlings, and molecular cloning of a full-length cDNA for a cotyledon-specific isoform. *J. Biochem.* **110**: 823-831.

Varner, J.E. 1993. Gene expression associated with tracheary element formation. *Plant Physiol.* **103**: 805-813.

von Heijne, G. 1986. Towards a comparative anatomy of N-terminsl topogenic protein sequences. *J. Mol. Biol.* **189**: 239-242.

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