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KEMENTERIAN PERDAGANGAN DALAM NEGERI
DAN HAL EHWAL PENGGUNA MALAYSIA,
BAHAGIAN HARTA INTELEK,
TINGKAT 27, 30 DAN 32,
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PATENT APPLICATION NO: PI 2001 5098

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16 September 2002

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CERTIFICATE OF FILING

APPLICANT	:	1) MALAYSIAN PALM OIL BOARD 2) CENTRE DE COOPERATION INTERNATIONALE EN RECHERCHE AGRONOMIQUE POUR LE DEVELOPPMENT (CIRAD)
APPLICATION NO.	:	PI 20015098
REQUEST RECEIVED ON	:	06/11/2001
FILING DATE	:	06/11/2001
AGENT'S/APPLICANT'S FILE REF.	:	SD/PAT/22403947/ZRS/CD

Please find attached, a copy of the Request Form relating to the above application, with the filing date and application number marked thereon in accordance with Regulation 25(1).

Date : 10/11/2001

.....

(Hasnon Bt. Alang Mohd Rashid)
for Registrar of Patents

To : **DATO' V.L. KANDAN**
M/s SHEARN DELAMORE & CO,
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Patents Form 1
PATENTS ACT 1983

REQUEST FOR GRANT OF PATENT
[Regulation 7(1)]

To: The Registrar of Patents
Patent Registration Office
Kuala Lumpur, Malaysia

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Application No. .....
Filing date: .....
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Applicant or Agent's file reference:
SD/PAT/2403947/ZRS/CD

THE APPLICANT(S) REQUEST(S) THE GRANT OF A PATENT IN RESPECT OF THE FOLLOWING PARTICULARS.

I. TITLE OF INVENTION

A NEW PROTEIN OF THE DEFENSIN FAMILY

II. APPLICANT(S)
(The data concerning each applicant must appear in this box or, if the space is insufficient, in the space below)

Name : 1. MALAYSIAN PALM OIL BOARD
2. CENTRE DE COOPERATION INTERNATIONALE EN RECHERCHE AGRONOMIQUE POUR LE DEVELOPPEMENT (CIRAD)

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Nationality : 1. A RESEARCH AND DEVELOPMENT BOARD INCORPORATED AND EXISTING UNDER THE LAWS OF MALAYSIA.
2. AN INSTITUTE INCORPORATED AND EXISTING UNDER THE LAWS OF FRANCE.

*Permanent residence or principal place of business: AS ABOVE

Additional Information

Delete whichever does not apply

III. INVENTOR(S)

The Applicant(s) is/are the inventor(s): Yes ☐ No ☒

If the Applicant(s) is/are not the inventor(s):

Name	Address
1. DR. CHEAH Suan Choo (Malaysian citizen)	6, Persiaran Institusi, Bandar Baru Bangi, 43000 Kajang, Selangor, Malaysia.
2. DR. James W. TREGEAR (British citizen)	CENTRE DE COOPERATION INTERNATIONALE EN RECHERCHE AGRONOMIQUE POUR LE DEVELOPPEMENT (CIRAD) CP/IRD Oil Palm Biotechnology Laboratory Centre IRD de Montpellier, BP 5045, 911 Avenue Agropolis, 34032 Montpellier, France.

A statement justifying the applicant's right to the patent accompanies this form: Yes ☒ No ☐

Additional information

IV. AGENT OR REPRESENTATIVE

The Applicant(s) has/have appointed a patent agent in the accompanying Form 17 (will follow) Yes ☒ No ☐

Patent Agent's Registration number: **PA 86/0003 and/or 86/0004**

Applicants have appointed ..... to be their common representative.

V. DIVISIONAL APPLICATION

This application is a divisional application: ☐

The benefit of the filing date ☐ and priority date ☐ of the initial application is claimed inasmuch as the subject matter of the present application is contained in the initial application identified below:

Initial Application number: .....

Date of filing of initial application: .....

VI. DISCLOSURES TO BE DISREGARDED FOR PRIOR ART PURPOSES

Additional information is contained in supplemental box:

(a) Disclosure was due to acts of the applicant or his predecessor-in-title ☐
Date of disclosure: ....

(b) Disclosure was due to the abuse of rights of applicant or his predecessor-in-title ☐
Date of disclosure: .....

A statement specifying in more detail the facts concerning the disclosure Accompanies this Form Yes ☐ No ☐

Additional information (if any)

VII. PRIORITY CLAIM (if any)

The priority of earlier applicant(s) is/are claimed as follows:-

Country*	Application no.	Filing date (dd/mm/yy)
-	-	-

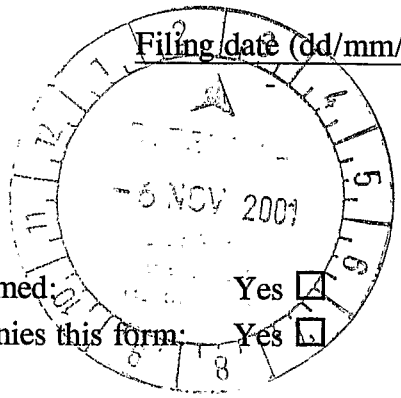
Symbol of the International Patent Classification:

If not yet allocated, please tick ☐

The priority of more than one earlier application is claimed: Yes ☐ No ☐

The certified copy of the earlier application(s) accompanies this form: Yes ☐ No ☐

If No, it will be furnished upon request.



*if the earlier application is a regional or international application, indicate the office with which it is filed

Additional information (if any)

VII. CHECK LIST

A. This application contains the following:

1.	Request (Form 1)	04	sheets
2.	description	15	sheets
3.	claim(s)	02	sheets
4.	abstract	01	sheets
5.	drawings (if any)	05	sheets
	TOTAL	27	

B. This Form, as filed, is accompanied by the items checked below:

a.	signed Form No. 17 (will follow)	☒
b.	declaration that inventor does not wish to be named in the patent	☐
c.	statement justifying applicant's right to the patent	☒
d.	statement that certain disclosure be disregarded	☐
e.	priority document (certified copy of earlier application)	☐
f.	cheque, ~~cash,~~ etc. ..... for the payment of application fee	☒
g.	other documents (specify)	☐

IX. SIGNATURE

.....

Name: DATO V L KANDAN
Regn No.: PA 86/0004

6 November 2001
.....
date

For official use:

1. Date application received: .....

2. Date of receipt of
correction,
later filed papers, or
drawings
completing the application. .....

DITERIMA
6 NOV 2001

20015098

A New Protein of the Defensin Family

The present invention relates to a new protein of the defensin family, which is useful in particular as a molecular marker of somaclonal variation events associated with the *mantled* homeotic flowering abnormality of oil palm.

Oil palm (*Elaeis guineensis* Jacq.) is a diploid monocotyledonous temporally dioecious species producing alternate cycles of male and female flowers.

In vitro micropropagation based on somatic embryogenesis has been used to carry out the multiplication of elite oil palm genotypes is described by RIVAL *et al.*, Plant Tiss. Cult. Biotechnol., 3, 74-83, 1996; however, the large scale use of this approach is hampered by the appearance of a somaclonal variant, known as *mantled* as described by CORLEY *et al.*, The Planter, 62, 233-240, 1986, which affects approximately 5-10% of palms cloned in this way as illustrated again by RIVAL *et al.*, Plant Tiss. Cult. Biotechnol., 3, 74-83, 1996. The *mantled* phenotype involves a feminisation of both male and female flowers: in the former, stamens develop as carpelloid structures, whilst in the latter, the staminodes (vestigial stamens) develop as pseudocarpel structures. In both cases, the petals appear to develop as sepalloid structures. In severe cases the flowers are sterile, although lesser affected female flowers may be fertilised to give *mantled* fruit.

In order to allow a more widespread use of *in vitro* micropropagation for oil palm, it is necessary to improve its reliability, by establishing an early detection method for the *mantled* abnormality. To this end, a detailed study has been carried out in order to identify molecular markers of the *mantled* phenotype.

Previous work revealed that the *mantled* abnormality is epigenetic in nature. Firstly, it was observed that reversion to a normal floral phenotype may occur in the field as described by DURAND-GASSELIN T. et al., Oléagineux 45, 1-11, 1990. Secondly, although the *mantled* abnormality is strongly transmitted through tissue culture, only a weak non-Mendelian transmission occurs via seeds as described by RAO V. and DONOUGH C.R., Elaeis, 2 199-207, 1990. Thirdly, ploidy, RAPD and AFLP studies have shown that the abnormal phenotype is unlikely to have resulted from any gross changes in genomic structure as described by RIVAL *et al.*, Plant Cell. Rep., 16, 884-887, 1997; RIVAL et al., Plant Breeding, 117, 73-76, 1998.

The present invention is based on the cloning and characterisation of a new oil palm gene, hereinafter referred as *EGAD1*. The *EGAD1* gene codes for a 77 amino acid polypeptide displaying strong similarities with plant defensins.

Northern studies of *EGAD1* gene expression show that in the intact oil palm plant, *EGAD1* transcripts accumulate mostly in inflorescence tissues, both normal and *mantled*, and show a peak of abundance during the early stages of development of the male inflorescence. On the other hand, in tissue culture, *EGAD1* transcripts are observed to accumulate throughout the regeneration procedure used for oil palm, a higher level of expression being observed at the nodular callus stage in cultures carrying the *mantled* abnormality than in those lacking it.

Thus, the expression of *EGAD1* provides an early marker of the *mantled* phenotype.

The present invention relates to an isolated polypeptide of the defensin family, wherein said polypeptide, hereinafter referred as EGAD1, has at least 70% sequence identity, preferably at least 80% sequence identity, and more preferably at least 90% sequence identity, with the polypeptide of SEQ ID NO: 2 in the enclosed sequence listing. The percent sequence identity is based on the entire region SEQ ID NO: 2 and is determined according to the CLUSTAL W program as illustrated by THOMPSON *et al.*, Nucl. Acids. Res., 22, 4673-4680, 1994, using the default settings.

The invention also comprises fragments of an EGAD1 polypeptide of the invention, comprising at least 5, preferably 10 contiguous amino-acids of said polypeptide.

The term "polypeptide" herein refers to a polymer of amino acid residues, resulting from the translation of a nucleic acid sequence, and possibly having post-translational modifications such as glycosylation, lipid attachment, hydroxylation, etc.

The present invention also relates to an isolated polynucleotide selected among:

a) a polynucleotide encoding an EGAD1 polypeptide; this includes for instance *EGAD1* genes, *EGAD1* cDNA, or *EGAD1* mRNA. A particular example is the cDNA of SEQ ID NO: 1;

b) a fragment of a polynucleotide a) at least 10, preferably at least 15, and more preferably at least 20 contiguous nucleotides thereof ;

c) a polynucleotide complementary to a polynucleotide a) or b) above.

The invention also comprises polynucleotides hybridising selectively under stringent conditions with any polynucleotide of the invention. This includes in particular nucleic acid probes for detecting a nucleic acid sequence encoding an EGAD1 polypeptide, as well as nucleic acid primers for amplifying a nucleic acid sequence encoding an EGAD1 polypeptide, or a portion thereof.

According to a preferred embodiment, a nucleic acid probe of the invention consists essentially of a polynucleotide of at least 10, preferably at least 15, and more preferably at least 20 nucleotides hybridising selectively under stringent conditions with a nucleic acid of SEQ ID NO: 1. Said probe may further comprise modifications, such as the addition of an appropriate label allowing its detection.

According to a preferred embodiment, a set of PCR primers of the invention consists of a fragment of at least 10, preferably at least 15, and more preferably at least 20 contiguous nucleotides of the polynucleotide of SEQ ID NO: 1, and a fragment of at least 10, preferably at least 15, and more preferably at least 20 contiguous nucleotides of the complementary of SEQ ID NO: 1. Appropriate primers can easily be designed by one of skill in the art from the sequence SEQ ID NO: 1.

The invention also encompasses any polynucleotide obtained from an oil palm genomic or cDNA library by PCR amplification with a set of primers of the invention.

The invention also comprises:

- a recombinant expression cassette comprising a polynucleotide of the invention encoding an EGAD1 polypeptide operably linked to a promoter;

- a recombinant nucleic acid vector having an insert consisting of a polynucleotide or of a recombinant expression cassette of the invention;

- a host cell transfected with a polynucleotide or a recombinant expression cassette of the invention;

- a transgenic plant comprising a recombinant expression cassette of the present invention.

The invention also provides a process for producing an EGAD1 polypeptide of the invention, wherein said process comprises culturing a host cell transfected with a recombinant expression cassette of the invention, and recovering the EGAD1 polypeptide from said culture. Said

polypeptide can be obtained from the cell lysate or from the culture medium using standard protein isolation techniques, in particular techniques suitable for isolation of defensins.

The invention also comprises polyclonal or monoclonal antibodies raised against an EGAD1 polypeptide of the invention.

The present invention also relates to the use of a polypeptide or a polynucleotide of the invention for detecting the *mantled* phenotype in an oil palm tissue culture.

More specifically, the present invention provides a method for detecting the *mantled* abnormality in an oil palm tissue culture, wherein said method comprises detecting the overexpression of the *EGAD1* gene encoding the EGAD1 polypeptide in said culture.

According to a preferred embodiment of the invention, the overexpression of the *EGAD1* gene is detected in an oil palm tissue culture at the callus stage.

The overexpression of the *EGAD1* gene is herein defined as a level of expression of said gene higher than the base-line expression level in a normal oil palm tissue culture.

Generally, the level of expression of the *EGAD1* gene in oil palm tissue culture bearing the *mantled* abnormality is at least 2 times higher, preferably at least 3 times higher, and more preferably at least 4 times higher, than its level of expression in a normal oil palm tissue culture.

The level of expression of the *EGAD1* gene can be determined by evaluating the amount of *EGAD1* transcripts. Techniques allowing the detection of an increased production of a mRNA or a protein are well known in the art. For instance, an increased production of a mRNA may be detected using the technique of northern blot analysis.

A strong association between the level of mRNA and the level of protein (in the form of defensins) accumulation in response to infections has been documented for defensin genes in other plant species.Terras et al (1995) reported a strong induction of expression of radish defensin gene in response to localised fungal infection. In the said study, the accumulation of both defensin transcripts and corresponding proteins was found to be strongly enhanced. Pennincky et al. (1996) reported a systemic increase in amounts of both defensin protein and mRNA in the host plant, *Arabidopsis thaliana* when infected by the pathogen *Alternaria brassicicola*.

Likewise, the level of expression of the EGAD1 gene may also be determined by evaluating the amount of EGAD1 polypeptide. An increased production of EGAD1 polypeptide may be detected by immunoassay using antibodies of the invention directed against said polypeptide.

The invention also encompasses reagents for practising the method of the invention. These reagents include in particular nucleic acid probes and sets of PCR primers defined above.

Reports of plant defensin genes conferring pathogen resistance through transgenesis have been reported. Gao et al. (2000) showed that by transforming an alfalfa defensin gene into potato, they were able to obtain resistance to the fungal pathogen *Verticillium dahliae* under field conditions. Terras et al. (1995), observed that when the radish Rs-AFP2 gene was transformed into tobacco under the control of the constitutive cauliflower mosaic 35S promoter, an enhanced resistance to the foliar fungal pathogen *Alternaria longpipes* was obtained.

The *EGAD1* gene and the EGAD1 polypeptide can also be used in the same way as previously described for other plant defensins, for the purpose of protecting plants against pathogens, in particular microorganisms such as fungi as described by THEVISSEN *et al.*, J. Biol. Chem. 271, 15018-15025, 1996; GAO et al. Nature Biotech., 18, 1307-1310 (2000); US Patent 6,121,436; .

The use of the EGAD1 gene and the EGAD1 polypeptide to protect plants against pathogens can be achieved by standard methods, for instance by transforming the plant to be protected with the sequence encoding an EGAD1 polypeptide, placed under the transcriptional control of an appropriate gene promoter.

The present invention will be further illustrated by the additional description which follows, which refers to the isolation of the *EGAD1* cDNA and the use of *EGAD1* sequences for detecting the *mantled* phenotype. It should be understood however that these examples are given only by way of illustration of the invention and do not constitute in any way a limitation thereof.

EXAMPLES

General methods

Plant material

Oil palm tissue cultures were established and maintained as previously described by PANNETIER *et al.*, Oléagineux, 36, 119-122, 1981. Two different clonal lines, LAB146 and LAB147, were used for differential display analysis. The LAB146 culture was obtained by direct cloning of a seed-derived palm and may therefore be assumed to carry little or no *mantled* abormality (i.e. it should generate either a low percentage of *mantled* regenerants or only normal clonal progeny). The LAB147 culture was obtained by recloning a *mantled* tissue culture-derived palm and can therefore be assumed to produce 100% abnormal regenerants as illustrated by RIVAL *et al.*, Plant Tiss. Cult. Biotechnol., 3, 74-83, 1996. For northern analysis, a range of cultures was used. Tissues were harvested corresponding to 3 developmental stages in the regeneration process: nodular callus, somatic embryos and 1 cm shoot apex-containing segments excised from leafy shoots. Inflorescence material was obtained from seed-derived and tissue culture-regenerated oil palms grown in Côte d'Ivoire and Malaysia. Root and leaf material was harvested from seed-derived greenhouse plants. For DNA extraction, leaf material from adult palms of several different genotypes (see below) was used.

Extraction and analysis of RNA and DNA

All standard cloning procedures used in this study were carried out essentially as described by SAMBROOK *et al.*, Molecular Cloning: A laboratory manual, Cold Spring Harbour Laboratory Press, Cold Spring Harbour, USA, 1989 unless otherwise indicated. Total RNA was extracted as described previously by CORRE *et al.*, L. Plant Sci., 117, 139-149, 1996. RNA gel electrophoresis and northern transfer were carried out using a NorthernMax-Gly® kit (AMBION CORPORATION). Membranes were hybridised with either ^{32}P-radiolabelled RNA probes synthesised using a Strip-EZ® RNA probe kit (AMBION CORPORATION) or ^{32}P-radiolabelled DNA probes obtained using the random priming method by FEINBERG and VOGELSTEIN, Analytical Biochemistry, 132, 6-13, 1983. Genomic DNA was extracted as previously described by RIVAL *et al.*, Plant Breeding, 117, 73-76, 1998 and analysed by Southern blotting and hybridisation using standard techniques.

Differential display analysis

Differential display analysis was carried out using the primers and PCR conditions described by MALHOTRA *et al.* as described in Nucl. Acids Res.,26, 854-856, 1998 with [-^{33}P]dATP as the radiolabel. After denaturing polyacrylamide gel electrophoresis, differential bands were excised and re-amplified by conventional PCR using the same primers as were used for the differential display amplifications. Re-amplified DNAs were blunt end cloned into the *Eco*RV site of the pBluescript SK- phagemid® (STRATAGENE). Several clones were sequenced for each marker re-amplification reaction and when more than one cDNA sequence was found to be cloned for a given marker, each cDNA was tested separately.

Construction and screening of cDNA library

A cDNA library was constructed from 1 cm shoot apex-containing segments excised from leafy shoots of the LAB147 culture grown for 3 weeks on medium containing 10-5 M benzylaminopurine (BAP). Screening was carried out by plaque hybridisation at 60°C in a buffer containing 5 x Denhardt's solution, 6xSSC, 4 µg/ml sheared salmon sperm DNA and 0.5% SDS.

EXAMPLE 1: CHARACTERISATION BY DIFFERENTIAL DISPLAY OF A CDNA FRAGMENT OVEREXPRESSED IN *MANTLED*-BEARING TISSUE CULTURES

Differences in mRNA accumulation between shoot apex-containing segments of the LAB146 (normal seed palm-derived) and LAB147 (abnormal clone-derived) cultures were studied using differential display, enabling the identification of differential bands specific to normal or abnormal material which were subsequently excised, re-amplified and cloned.

One of the abnormal-specific cDNA fragments obtained, provisionally named m5B, was shown by Northern hybridisation to correspond to an mRNA of ca. 0.6 kb which accumulates at higher levels in LAB147 shoot apex-containing segments than in those obtained from the LAB146 culture, as shown in Figure 1.

Figure 1 shows a northern hybridisation analysis of transcript accumulation using a ^{32}P-radiolabelled RNA probe synthesised from the m5B differential display clone containing part of the *EGAD1* cDNA sequence. The m5B (*EGAD1*) probe was hybridised to total RNA extracted

from oil palm tissue cultures and organs of intact plants (upper panel). As a reference, a control hybridisation to oil palm ^{32}P-radiolabelled 25S ribosomal DNA probe was also performed (lower panel). Two different tissue cultures were examined: LAB146, a normal seed palm-derived culture (labelled "SPDC") and LAB147, an abnormal (*mantled*) clone-derived culture (labelled "ACDC"). Other abbreviations are as follows:

- INFLO, spikelets of female inflorescences;

- N, normal inflorescence (25 cm in length);

- A, abnormal inflorescence (30 cm in length).

In a preliminary characterisation of the tissue specificity of the m5B mRNA, it was found that the difference in accumulation of the 0.6 kb transcript between the LAB146 (normal) and LAB147 (abnormal) cultures was even more marked for calli than for shoot apex-containing segments. Little or no difference in signal intensity was observed for somatic embryos.

In whole plants, the 0.6 kb transcript was found to be present in both normal and abnormal female inflorescences (25 cm and 30 cm respectively in length) harvested from clonal palms, similar levels being observed in each case.

No m5B-specific signal was observed for leaves or roots of intact seedling plants, although a longer exposure of the autoradiograph shown produced a faint signal for roots.

Overall, it can be concluded from the Northern hybridisation shown in Figure 1 that transcripts corresponding to the m5B cDNA accumulate principally in inflorescence tissues in the intact plant, as well as in tissue cultures in an apparently *mantled*-dependent fashion.

EXAMPLE 2: ISOLATION AND CHARACTERISATION OF THE FULL LENGTH *EGAD1* CDNA

The cDNA insert in the m5B clone was used as a probe to screen an oil palm cDNA library prepared from shoot apex-containing segments of leafy shoots grown on BAP-containing medium, allowing the purification and sequencing of 6 positive clones containing the previously determined m5B nucleotide sequence. A database search revealed that each of the cDNAs thus obtained codes

for a cysteine-rich polypeptide sharing close similarities with the plant defensin (or -thionin) proteins as described by TERRAS *et al.*, Plant Cell, 7, 573-588, 1995. Although slight variations in size were observed between the cloned cDNAs, only one (m5B-15) displayed a divergent nucleotide sequence, the others all being base identical. Since the m5B-15 cDNA diverges from the other 5 cDNAs at only 3 nucleotide positions, we believe that it might represent a different allele of the same gene locus. This hypothesis is consistent with our genomic Southern data (see Example 3 below). The longest cloned cDNA obtained, m5B-7, was selected for use in subsequent experiments and renamed *EGAD1*. The nucleotide sequence of the 535 bp *EGAD1* cDNA and the deduced sequence of the polypeptide for which it codes are represented respectively as SEQ ID NO: 1 and SEQ ID NO: 2 and are also shown in Figure 2.

Figure 2 also shows the nucleotide sequence of the *EGAD1* (m5B-7) cDNA and the deduced sequence of the encoded polypeptide. The position of the putative signal peptide sequence is indicated by underlining. The position of the putative translation termination codon is denoted by an asterisk.

EGAD1 codes for a 77 amino acid polypeptide of predicted size 5.3 kD. The *EGAD1* cDNA sequence shown in Figure 2 is assumed to contain a full length coding region, since it includes a putative translation initiation codon which is conserved amongst plant defensins and which is preceded in the same reading frame by a TAG stop codon 12 bp further upstream. The polypeptide encoded by the *EGAD1* gene contains a putative signal peptide sequence of 30 amino acids which is likely to be responsible for targeting the nascent polypeptide into the secretory pathway. In order to examine amino acid sequence conservation between the EGAD1 precursor and those of other defensin/-thionin proteins, a CLUSTAL alignment was carried out. The EGAD1 precursor sequence was aligned, using the CLUSTAL W program as described by THOMPSON *et al.*, Nucl. Acids Res., 22, 4673-4680, 1994 with those of its 3 closest published relatives, namely the products of the genes *PPT* from *Petunia inflata* as illustrated by KARUNANANDAA *et al.*, Plant Mol. Biol., 26, 459-464, 1994, *J1-1* from *Capsicum annuum* as described by MEYER *et al.*, Plant Physiol., 112, 615-622, 1996 and *P322* from potato as described by STIEKEMA *et al.*, Plant Mol. Biol., 11, 255-269, 1988.

Figure 3 shows the alignment of the deduced EGAD1 protein sequence with those encoded by the genes *PPT* from *Petunia inflata*, j1-1 from *Capsicum annuum* and P322 from potato. Asterisks indicate residues which are absolutely conserved in all 4 polypeptide sequences, whilst single and double dots respectively denote conservations within "weaker" or "stronger" groups as defined

within the CLUSTAL W program. The position of the putative translation termination codon is indicated by an asterisk.

The closest identified relative of EGAD1 is PPT, the two respective sequences sharing 63.6% identical residues. It is interesting to note that the positions of the 8 cysteine residues are absolutely conserved between all 4 polypeptides, strongly suggesting that they all share the same secondary structure. Defensins are thought to play a role in pathogen defence and in some cases have been shown to exert an antifungal action, probably brought about by electrostatic interaction with hyphal cell membranes as described by THEVISSEN *et al.*, J. Biol. Chem. 271, 15018-15025, 1996.

EXAMPLE 3: ESTIMATION OF THE NUMBER OF *EGAD1*-LIKE GENE LOCI PRESENT IN THE OIL PALM GENOME

Oil palm genomic DNA was extracted from leaves of 3 different adult oil palms, X, Y and E, of different genotype in each case (NB palms X and E were used to obtain the tissue cultures X1 and LAB146 respectively). DNAs were digested with 3 different restriction enzymes, Southern blotted and hybridised to the *EGAD1* cDNA.

Figure 4 shows a Southern hybridisation of a ^{32}P-radiolabelled *EGAD1* cDNA probe to oil palm genomic DNA digests from the three genotypically different oil palms, X, Y and E,. The restriction enzymes used and size marker migration positions are indicated.

These results suggest that the *EGAD1* gene occurs as a single copy per haploid genome. All 3 restriction enzymes used (none of which cuts the *EGAD1* cDNA) were found to produce only one hybridising fragment, except in the case of *Eco*RI when used with DNA of the Y genotype; in this case, a doublet was observed. One possible explanation for the latter result is that it might be caused by a difference in restriction pattern between allelic regions in or bordering the *EGAD1* gene locus in the Y genome.

EXAMPLE 4: INVESTIGATION OF THE *MANTLED*-DEPENDENT EXPRESSION OF *EGAD1* IN OTHER OIL PALM GENOTYPES AND CULTURES

Since the northern hybridisation data shown in Figure 1 revealed a strong differential accumulation of *EGAD1* transcripts at the callus stage of tissue culture regeneration, we investigated this phenomenon further. The N and A cultures used for the differential display and preliminary expression analysis were not of identical genotype; thus it was important to check that the difference in transcript levels seen was not simply attributable to differences in genetic background. We therefore investigated *EGAD1* transcript accumulation in genotypically identical cultures differing only in their *mantled* status, namely the cultures X1 (normal), X2 (intermediate) and X3 (abnormal). These cultures were initiated from respectively a normal seed-derived palm, a normal regenerant palm previously cloned from the seed-derived palm and an abnormal regenerant palm again previously cloned from the same original palm. The X1, X2 and X3 cultures were all initiated at the same time, so as to eliminate any possible effects due to culture age. In terms of abnormality, the X2 culture can be considered as representing an intermediate situation in that it has been initiated from a normal palm, but consists of cells which have undergone an extra round of tissue culture compared with the X1 culture. In the same experiment, in order to check the reproducibility of *EGAD1* expression between different cultures obtained from a given starting material, we analysed callus lines N' (LAB148) and A' (LAB149), which were respectively initiated from the same palms as the LAB146 and LAB147 cultures. Figure 6 shows the northern hybridisation profiles obtained with each callus line using the *EGAD1* probe. The hybridisation profiles of the N' and A' samples are consistent with those shown in Figure 1 for the N and A lines, thus confirming the reproducibility of these results between cloning experiments and providing further evidence that the differential *EGAD1* gene expression patterns observed may be associated with the *mantled* abnormality. This hypothesis is further strengthened by the hybridisation profile observed for the X1, X2 and X3 cultures, which differ in their *mantled* status within a common genotypic background. As expected, the X3 (abnormal) culture produces a dramatically stronger signal than the X1 (normal) culture. It is moreover interesting to note that the X2 (intermediate) culture produces a signal greater than that of X1, but weaker than that of X3, presumably reflecting the extra cycle of tissue culture "history" which it carries compared with the X1 line.

The above data confirm that *EGAD1* gene expression is affected by epigenetic factors which prevail in tissue culture, resulting in increased transcript accumulation in abnormal clonal lines at the callus stage. The reproducibility of *EGAD1* gene expression profiles between cultures

established at different times from the same palm has been demonstrated. Most importantly, studies performed on the genotypically homegeneous X1/X2/X3 cultures show that *EGAD1* gene expression provides a sensitive marker for the presence of the *mantled* abnormality at the callus stage.

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Lys Phe Gln Gly Thr Cys Leu Arg Glu Ser Asn Cys Ala Asn Val Cys
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	50					55					60				
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65					70					75					

CLAIMS

1) An isolated polypeptide of the defensin family, hereinafter referred as EGAD1, having at least 70% sequence identity with the polypeptide of SEQ ID NO: 2.

2) A fragment of an EGAD1 polypeptide of claim 1, comprising at least 5 contiguous amino-acids of said polypeptide.

3) An isolated polynucleotide selected among:

a) a polynucleotide encoding an EGAD1 polypeptide of claim 1;

b) a fragment of a polynucleotide a) comprising at least 10 contiguous nucleotides;

c) a polynucleotide complementary of a polynucleotide a) or b) above;

d) a polynucleotide hybridising selectively under stringent conditions with a polynucleotide a) b) or c) above.

4) A polynucleotide of claim 3 selected among:

a) the cDNA of SEQ ID NO:1;

b) a polynucleotide comprising at least 10 contiguous nucleotides of SEQ ID NO: 1;

c) a polynucleotide complementary of a polynucleotide a) or b) above;

d) a polynucleotide hybridising selectively under stringent conditions with a polynucleotide a), b) or c) above.

5) The use of a polypeptide of any of claims 1 or 2 for detecting the *mantled* phenotype in an oil palm tissue culture.

6) The use of a polynucleotide of any of claims 3 or 4 for detecting the mantled phenotype in an oil palm tissue.

7) A method for detecting the *mantled* abnormality in an oil palm tissue culture, wherein said method comprises detecting the overexpression of the *EGAD1* gene, encoding the EGAD1 polypeptide of claim 1, in said culture.

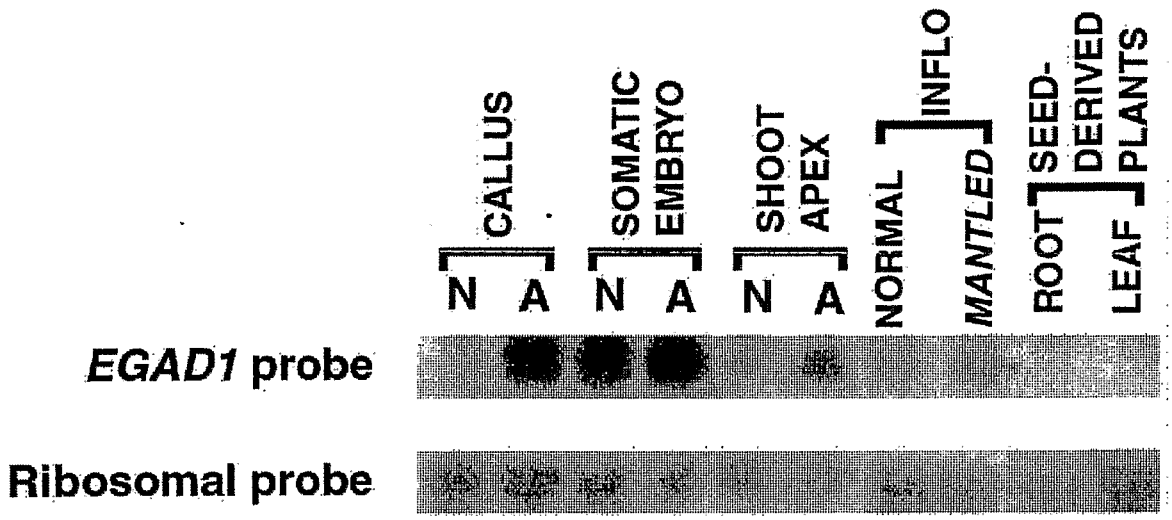
8) A method of claim 6, wherein the overexpression of the *EGAD1* gene is detected in an oil palm tissue culture at the callus stage.

9) A method of claim 7, wherein the overexpression of the *EGAD1* gene is detected by evaluating the amount of *EGAD1* transcripts.

A New Protein of the Defensin Family

ABSTRACT

The invention relates to new protein of the defensin family , its isolated polypeptide and an isolated polynuceotide encoding it. The invention further relates to the uses of the polynucleotide and or polypeptide, including its use to detect the mantled abnormality of oil palm.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ROOT
SEED-DERIVED
LEAF
PLANTS
N A N A N A
EGAD1 probe
Ribosomal probe

FIG. 1

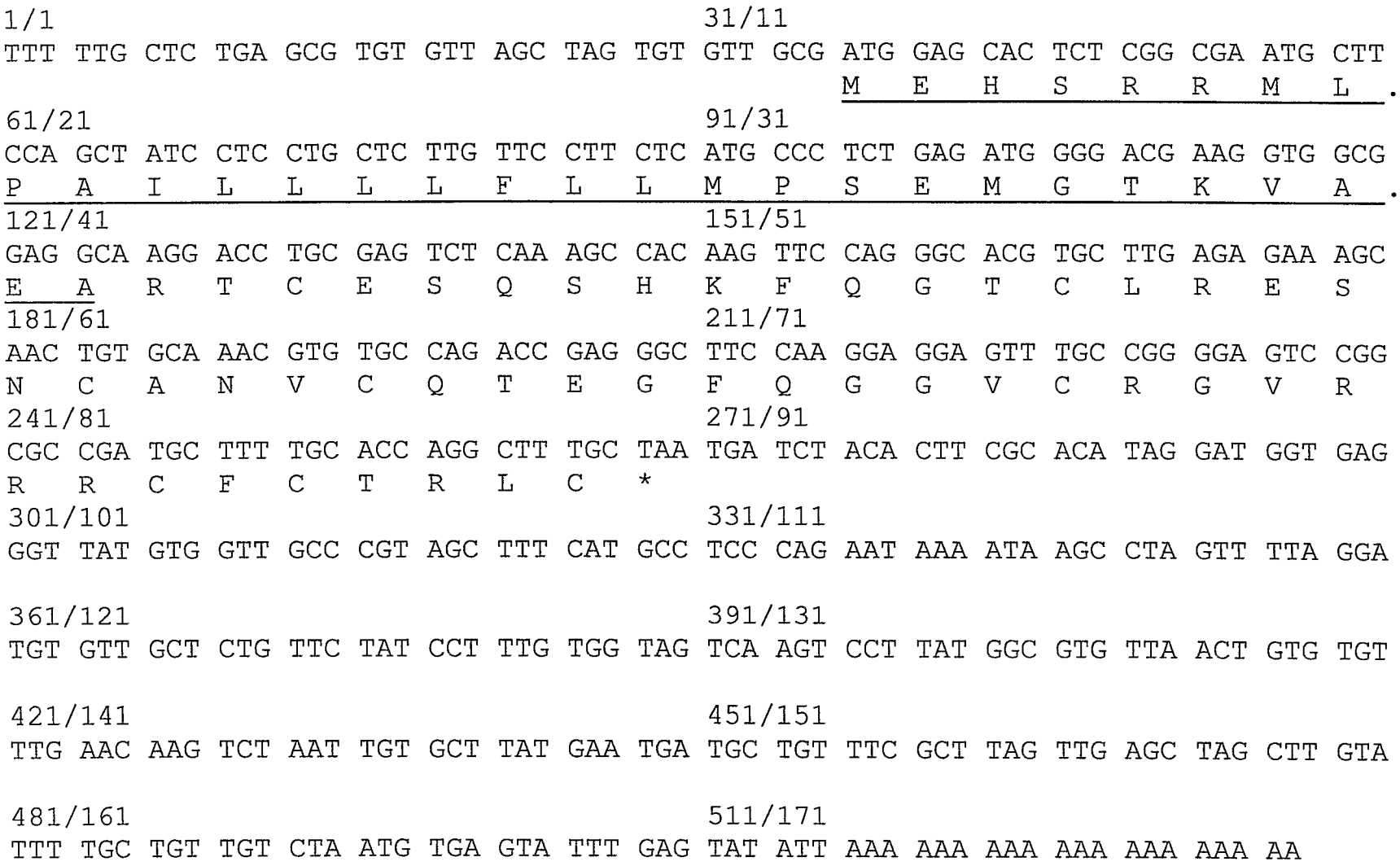
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FIG. 2

```
‡
PPT             MGRSIRLFATFFLIAMLFLSTEMGPMTSAEARTCESQSHRFHGTCVRESNCASVCQTEGF
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j1-1            MAGFSKVIATIFLMMMLVFATGM----VAEARTCESQSHRFKGLCFSKSNCGSVCHTEGF
EGAD1           MEHSRRMLPAILLLLFLLMPSEMG-TKVAEARTCESQSHKFQGTCLRESNCANVCQTEGF
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PPT             IGGNCRAFRRRCFCTRNC
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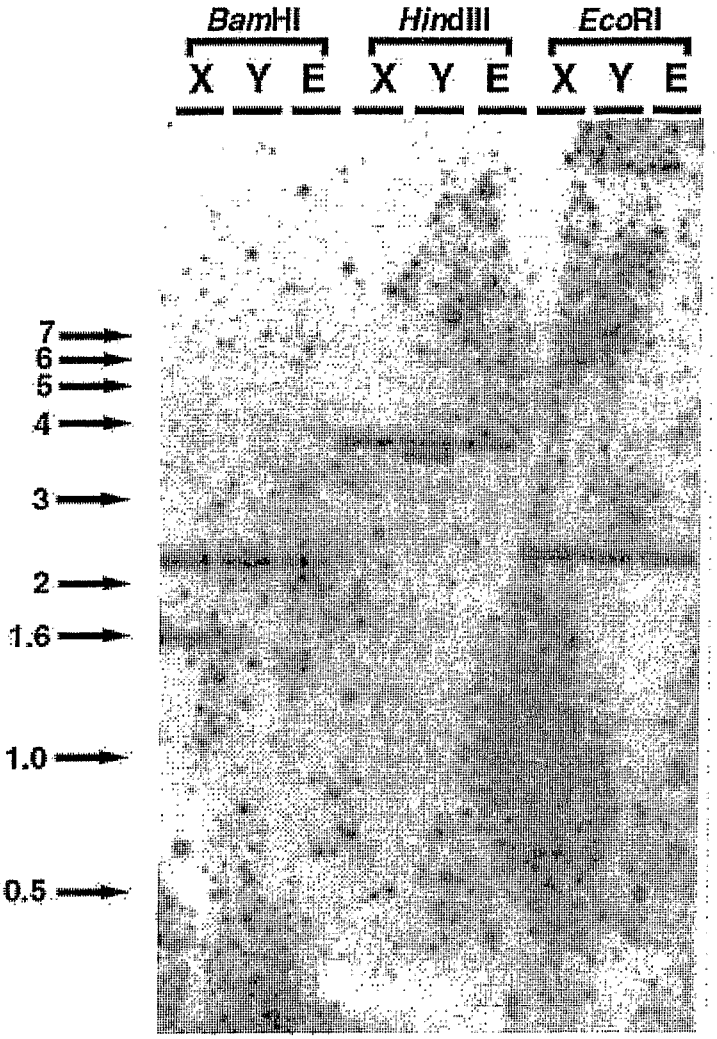
FIG. 3



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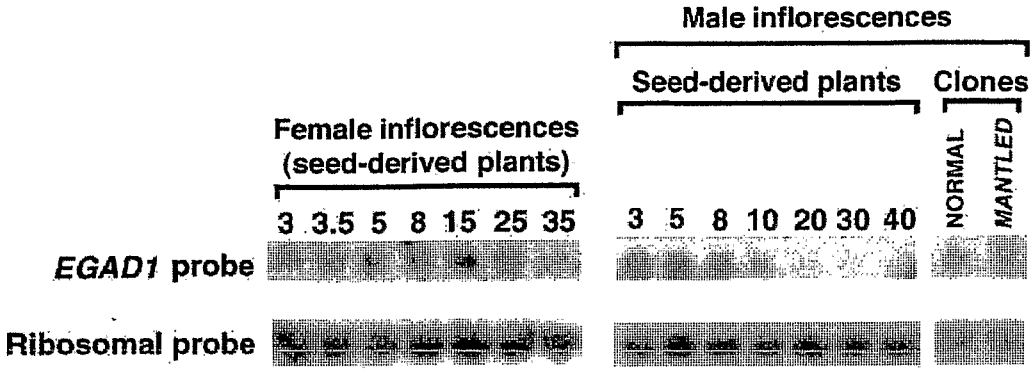
Male inflorescences
Seed-derived plants
Clones
Female inflorescences
(seed-derived plants)
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MANTLED
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3 5 8 10 20 30 40
EGAD1 probe
Ribosomal probe

FIG. 5

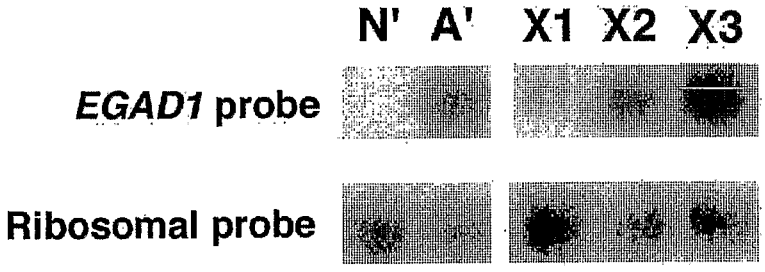
N' A' X1 X2 X3
EGAD1 probe
Ribosomal probe

FIG. 6