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Contrasting globulin and cysteine proteinase gene expression patterns reveal fundamental developmental differences between zygotic and somatic embryos of oil palm

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Summary Oil palm (*Elaeis guineensis* Jacq.) somatic embryos differ from zygotic embryos in that they accumulate only small amounts of storage proteins. We compared the balance between deposition and degradation of storage proteins during zygotic or somatic embryogenesis and germinative growth in the two types of embryos. During mid to late zygotic embryogenesis, storage proteins accumulated and globulin 7S (*GLO7A*) gene transcripts were detected, whereas neither protease activity nor cysteine proteinase (*CPR*) gene transcripts were detected. Globulin degradation occurred after 8 days of in vitro germination in zygotic embryos and was accompanied by a decrease in *GLO7A* transcripts. Transcripts of three cysteine proteinase genes of the papain family were detected as early as Day 2 of in vitro germination. Several proteolytically active protein bands were identified by zymography, and CPR-like proteins were detected with an antibody raised against the *Vicia sativa* L. cysteine proteinase CPR1. Protease activities and CPR-like proteins were observed from Day 8 onward when globulin degradation occurred.

During somatic embryogenesis and subsequent germinative growth, only small amounts of storage proteins accumulated, even though *GLO7A* transcripts were detected. Two of the three cysteine proteinase genes were expressed throughout both somatic embryogenesis and germinative growth. Protease activities and CPR-like protein species were detected in somatic embryos at several developmental stages. In contrast to zygotic embryogenesis, the accumulation of globulins and their subsequent mobilization appear to be concomitant processes during somatic embryogenesis, which could explain the low accumulation of storage proteins in somatic embryos.

Keywords: Elaeis guineensis, embryogenesis, germination, protease, storage protein.

Introduction

Somatic embryogenesis is the process by which somatic cells develop into a bipolar structure resembling a zygotic embryo, without vascular connections to the original tissue (von Arnold et al. 2002). Somatic embryogenesis has been used as a model for studying the regulation of embryo development (Zimmerman 1993), and for large-scale propagation of plants of commercial interest, including angiosperms such as coffea (Etienne et al. 2006) and banana (Rout et al. 2000), and gymnosperms such as pine and spruce (Misra 1994, Merkle and Dean 2000). A wide range of work has been undertaken to compare zygotic and somatic embryo development including morphological and gene expression studies in carrot (Dodeman et al. 1997) and pine (Rodriguez et al. 2006, Tereso et al. 2007), and proteomic analysis (Winkelmann et al. 2006) and the analysis of cell cycle events in cyclamen (Schmidt et al. 2006). Among the main differences between zygotic and somatic embryogenesis are that somatic embryos depend on somatic cells to acquire embryogenic competence, lack endosperm differentiation, have retarded or no suspensor development and exhibit neither desiccation, maturation nor dormancy phases (Feher et al. 2003).

The maturation phase, which is often incomplete in somatic embryos, determines the vigor of the regenerated plantlets and the feasibility of storing embryos obtained by large-scale propagation (Merkle et al. 1995). In developing seeds, this phase is characterized by the deposition of storage proteins in both embryo and endosperm. The existence of an endosperm is a major difference between zygotic and somatic embryos. Storage proteins, which are the main proteins accumulated, are considered to be markers of the maturation phase (Galau et al. 1991). Deposition of storage proteins has been reported in somatic embryos for several angiosperm and gymnosperm species including alfalfa (Lai et al. 1995), maize (Duncan et al. 2003) and white spruce (Misra et al. 1993). However, storage protein composition and quantity often differ between somatic embryos and zygotic embryos with smaller amounts reported in somatic embryos of cotton (Shoemaker et al. 1987), alfalfa (Krochko et al. 1994) and carrot (Dodeman et al. 1998).

Mobilization of stored proteins occurs during seed germination to supply the young seedling with the reduced nitrogen needed for growth and development (Callis 1995). Germina-

tion can be divided into two main steps: early germination and germinative growth (Bewley 1997). Early germination starts with seed imbibition and ends with the elongation of the embryonic axis. During germinative growth, also termed early seedling growth, stored reserves are mobilized (Bewley 1997). Protein degradation is catalyzed by proteases, among which the cysteine proteinase (CPR) families papain (C1) and legumain (C13) play a prominent role (Müntz et al. 2001). Several cysteine proteinases have been identified in rice (oryzains α, β and γ; Watanabe et al. 1991), maize (*mir1*, *mir2* and *mir3*; Pechan et al. 1999) and *Vicia sativa* L. (CPR1, CPR2 and proteinase A; Becker et al. 1994, 1997). Depending on the plant species, hydrolysis is catalyzed by proteinases initially stored in an inactivated form in protein bodies in the dry seed or by enzymes synthesized de novo when germination occurs, or both (Müntz et al. 2001). Few data are available on the occurrence of proteinase activity in somatic embryos. In carrot, several cysteine proteinase genes were identified that showed developmentally regulated expression and activity during somatic embryogenesis (Mitsuhashi et al. 2004); however, their relationship with protein degradation was not investigated and comparative studies with the zygotic embryo were not reported.

Somatic embryogenesis is used to propagate elite plants from the economically important species oil palm (*Elaeis guineensis* Jacq.; Duval et al. 1997). Micropropagation using embryogenic suspension cultures allows the large-scale production of oil palm somatic embryos (Aberlenc-Bertossi et al. 1999). The comparison of oil palm somatic embryos produced in vitro with their zygotic counterparts *in planta* has highlighted biochemical differences between these embryo types during maturation. Zygotic embryo maturation is characterized by the deposition of storage proteins identified as 7S globulins and 2S albumins (Morcillo et al. 1997). Amounts of 7S globulins were 80 times lower in somatic embryos than in zygotic embryos (Morcillo et al. 1998). Low globulin accumulation in somatic embryos was attributed at least in part to low accumulation of transcripts of the 7S globulin gene *GLO7A* (Morcillo et al. 2001). However, observed decreases in soluble globulin content during somatic embryo development suggested that hydrolysis of storage proteins also occurs (Morcillo et al. 1998).

In this study, we investigated the balance between accumulation and degradation of globulins in oil palm somatic embryos using zygotic embryos as a reference. We observed that, in zygotic embryos, protease activities and cysteine proteinase transcript accumulation were concomitant with globulin degradation and were specific to the germinative growth phase. In somatic embryos, protease activity was present throughout embryogenesis and germinative growth, and likely explains the low accumulation of storage proteins in these embryos.

Materials and methods

Plant material

Oil palm zygotic embryos were excised from hybrid *dura* × *pisifera* seeds (genetic reference C1001) obtained after controlled pollination, harvested between 89 and 160 days post anthesis (DPA) and provided by INRAB in Benin. Dry seeds, imbibed for 5 days in water containing 0.1% benlate, were broken, kernels were removed and sterilized in 2.5% (v/v) sodium hypochlorite for 10 min. Kernels were rinsed with sterile water, and zygotic embryos were aseptically removed and germinated in vitro on a basal medium containing Murashige and Skoog's macroelements as modified by Rabéchault and Martin (1976), Nitsch's microelements (1969), Morel and Wetmore's vitamins (1951), Fe EDTA, 100 mg l^{-1} sodium ascorbate, 100 mg l^{-1} *myo*-inositol and 30 g l^{-1} sucrose. Before autoclaving, the pH was adjusted to 5 and 2 g l^{-1} phytagel was added. Cultures were grown at 27 °C in a 12-h photoperiod at 45 μmol m^{-2} s^{-1}. Five to 50 embryos were harvested from immature and dry seeds after 1, 2, 4, 8, 12 or 16 days of germination, and fresh masses were determined gravimetrically from three measurements, each of 20 embryos. Embryos were immersed and stored in liquid nitrogen until further analysis.

Somatic embryos were regenerated from embryogenic suspension cultures according to Aberlenc-Bertossi et al. (1999). Somatic embryos were plated on basal medium supplemented with 5 μM benzyladenine for 1 week and then subcultured on hormone-free medium. The pH was adjusted to 5, and the medium was gelled with 8 g l^{-1} agar. Somatic embryos were harvested at three developmental stages: Stage I, 8 days after plating, when embryos were about 1 mm long; Stage II, 28 days after plating, when embryos were 2–3 mm long; and Stage III, 41 days after plating, when embryos were 4–5 mm long. Somatic embryos were also harvested after 1, 2, 4, 8, 12 and 17 days of in vitro germination. Fresh masses were determined gravimetrically from three measurements, each of 20 embryos.

Isolation and characterization of cysteine proteinase cDNAs

Three cysteine proteinase cDNA clones were identified in the oil palm expressed sequence tag (EST) collection established in our laboratory (Jouannic et al. 2005), namely *CPRZ* (Accession no. EF622019), *CPRF* (EF622020) and *CPRS1* (EF62-2021). Missing cDNA regions were obtained using a smart RACE cDNA amplification kit (Clontech) in conjunction with total RNA isolated from zygotic embryos harvested after 4, 8 and 12 days of in vitro germination. The PCR primers for RACE were designed according to the recommendations of the kit manufacturer. The PCR products were ligated into pGEM-T Easy (Promega) and transformed into *Escherichia coli* JM 109 strain. Plasmid DNA was isolated from individual transformants using Qiaquick PCR purification kit (Qiagen) and sequenced (GenomeExpress, Meylan).

Gene expression analysis by semi-quantitative RT-PCR

Total RNA was isolated from zygotic and somatic embryos using the RNeasy plant mini kit (Qiagen), except for immature zygotic embryos from dry seeds germinated in vitro for 2 and 4 days which were extracted using the RNeasy lipid tissue kit (Qiagen). Reverse transcription was performed from 1 μg RNA with the kit Improm II (Promega) and PCR analyses were performed under the following conditions: 1 cycle at

94 °C for 3 min, 25 cycles for 30 s at 94 °C, 50 °C for 30 s, 72 °C for 1 min and, finally, 1 cycle at 72 °C for 10 min. Primers were designed to allow specific gene amplification (Table 1). The presence of genomic DNA in samples was checked by the presence of bands obtained after PCR with actin primers designed on both sides of an intron region. The sequences of the RT-PCR products were determined by cloning and sequencing to check the specificity of the amplifications.

Protein extraction and quantitation

A protein extraction protocol was adapted from Solomon et al. (1999). Embryos were ground in liquid nitrogen and the resulting powder was dispersed in a buffer containing 20 mM Tris, pH 7.8, 20 mM NaCl and 1 mM EDTA. Cells and insoluble matter were removed by centrifuging at 4 °C at 16 000 *g* for 10 min, and extracts were desalted through a PD 10 column (Amersham). Protein contents were determined by the Bradford method (Bradford 1976) with Biorad dye reagent (Biorad, Hercules, CA).

Protein electrophoresis, immunoblotting and protease activity assay

Proteins were analyzed by SDS-PAGE (12.5% polyacrylamide) according to the method of Laemmli. Five to 20 µg of soluble protein was loaded per lane. Gels were stained with Coomassie Brilliant Blue R250. Immunodetection was undertaken after separation of the proteins on SDS-PAGE (10% polyacrylamide) by electrophoretically transferring the proteins onto a nitrocellulose membrane (Immobillon P, Millipore). Blots were blocked overnight in 0.1 M PBS, 0.14 M sodium chloride, pH 7.4, containing 3% gelatine and 0.25% Triton X-100. The CPR1-like proteins were detected with a specific antibody raised against recombinant CPR1 cysteine proteinase of *Vicia sativa* L. (Becker et al. 1994). Blots were incubated, first, with CPR1-antibody diluted 1:2000 and, second, with the anti-Immunoglobulin G, rabbit alkaline phosphatase conjugate (Sigma) diluted 1:500. After incubation, blots were stained with nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) (Biorad).

For the in-gel protease activity assay, SDS-polyacrylamide gels (7.5% polyacrylamide) were copolymerized with gelatin 0.05% (w/v). Five to 20 µg of soluble protein was loaded per lane. Samples were not heated before loading for zymographic analysis. After electrophoresis, the gel was washed three times in water containing 0.25% Triton X-100 and incubated in McIlvaine buffer, pH 4, containing 2 mM dithiothreitol overnight at 27 °C to restore protease activity. Cysteine proteinase active bands were identified by the addition of 50 µM of the specific cysteine proteinase inhibitor L-*trans*-epoxysuccinyl-leucylamido(4-guanidino) butane (E64) (Sigma-Aldrich) to the overnight incubation buffer for one set of gels. Gels were briefly rinsed and stained with Coomassie Brilliant Blue R250. Protease activity was visualized as clear bands on a blue background.

Table 1. Sequences of oil palm primers used for polymerase chain reactions. Primers were designed to allow specific gene amplification.

Gene	Primer	Sequence
GLO7A		
Sense	GLOS2	5′-ATCGTTCCTGCTGGCCACCCA-3′
Antisense	GLOAS	5′-CGTTCCATCTCAGAAGCCGCC-3′
CPRZ		
Sense	FZS1	5′-AACCAGGGCTGCAATGG-3′
Antisense	ZAS	5′-CAAGTCAGCAAAGACGTTC-3′
CPRF		
Sense	FZS1	5′-AACCAGGGCTGCAATGG-3′
Antisense	FAS	5′-GCGCACAGGCGTCGAGTACC-3′
CPRS1		
Sense	CPRS1S1	5′-GGTGGTGAGTGGTTTCAGG-3′
Antisense	CPRS1AS1	5′-GGATTGATCTCAACTGTATTC-3′
Actin		
Sense	ACT1S1	5′-CTTGCTCCAAGCAGCATGAA-3′
Antisense	ACT1AS1	5′-AGAAGCACTTCCGGTGCACG-3′
EF1α		
Sense	EF1S2	5′-GGTGTGAAGCAGATGATTTGC-3′
Antisense	EF1AS2	5′-CCTGGATCATGTCAAGAGCC-3′

Results

Embryogenesis and germination

Zygotic embryos were extracted during *in planta* embryogenesis at various developmental stages following anthesis. Before 89 days after pollination (DAP), the small size of the embryos precluded their isolation. From 89 to 162 DAP, zygotic embryo fresh mass was relatively constant at around 4 mg (Figure 1A). Maturity and fruit shedding were reached at around 160–180 DAP. Toward the end of embryo development, fresh mass decreased to 1.7 mg as the seed dried. Following seed imbibition, excised embryos were germinated in vitro. Two days after germination (DAG), embryo fresh mass increased to around 4.5 mg and stabilized until Day 4. After Day 4, the embryos started to elongate and fresh mass increased to 10.8 mg at Day 8. After 16 days of in vitro germination, growth continued and embryo fresh mass reached 43.2 mg. According to Bewley (1997), Days 0–4 correspond to the germination stage, and Days 4–16 correspond to the post-germination growth stage, referred to hereafter as germinative growth.

Under our culture conditions, somatic embryo development resulted in morphologically variable embryos. The variation in fresh mass was greater in somatic embryos than in zygotic embryos, particularly at Day 17 when some embryos showed heterogeneous growth. After 1 month of culture in liquid medium without hormones, embryogenic suspension cultures were plated onto semi-solid medium for embryogenesis. At 8 days after plating (DAPL), somatic embryos were about 1 mm. Embryo fresh mass increased progressively from 6.7 mg after 28 days of embryogenesis to 13.9 mg after 41 days (Figure 1B). Embryos were then germinated in vitro and after 17 days, fresh mass reached 70.4 mg. Roots and shoots were observed after 4 and 17 days of in vitro germination, respec-

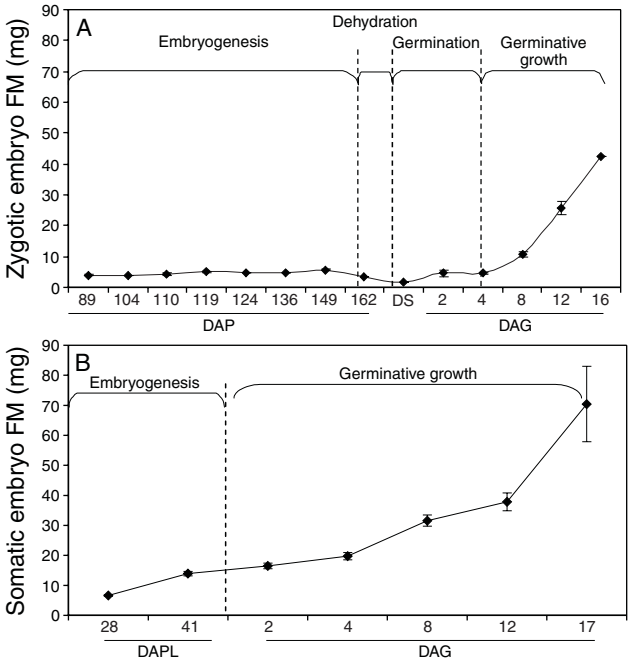

Figure 1. Fresh masses (FM) of oil palm (*Elaeis guineensis*) zygotic and somatic embryos at different developmental stages. (A) Zygotic embryos harvested a given number of days after pollination (DAP), excised from dry seed (DS) or harvested a given number of days after in vitro germination (DAG). (B) Somatic embryos harvested a given number of days after plating (DAPL), and after in vitro germination (DAG). Fresh masses were determined gravimetrically from three measurements each of 20 embryos.

tively. Fully hydrated somatic embryos do not undergo a germination stage; however the constant and progressive increase in their fresh mass during in vitro germination is analogous to the zygotic embryo's germinative growth stage and is hereafter referred to as germinative growth.

Storage protein patterns

Analysis of the electrophoretic pattern of total proteins isolated from zygotic embryos revealed the presence of bands with molecular masses of 19–22 kDa and 45–65 kDa (Figure 2A), corresponding to 2S albumins and 7S globulins, respectively. In embryos from dry seeds and in embryos between 90 and 165 DAP and up to 1 or 2 DAG, both globulins and albumins accumulated in large amounts. From 1 to 4 DAG, the amount of storage protein decreased slightly (Figure 2A). Hydrolysis of storage proteins was first observed between Days 4 and 8 of the germinative growth period. By Day 16, storage proteins were almost entirely degraded. In somatic embryos, no accumulation of storage protein was detected either during embryogenesis or during the germinative growth phase (Figure 2B).

Transcripts of the *GLO7A* gene were detected in embryos excised from dry seeds and throughout zygotic embryogenesis until Day 4 of in vitro germination (Figure 3A). From Day 8, *GLO7A* transcripts were no longer detected, concomitantly with the observation of storage protein degradation. In contrast, *GLO7A* transcripts were detected in somatic embryos throughout embryogenesis and germinative growth (Figure 3B).

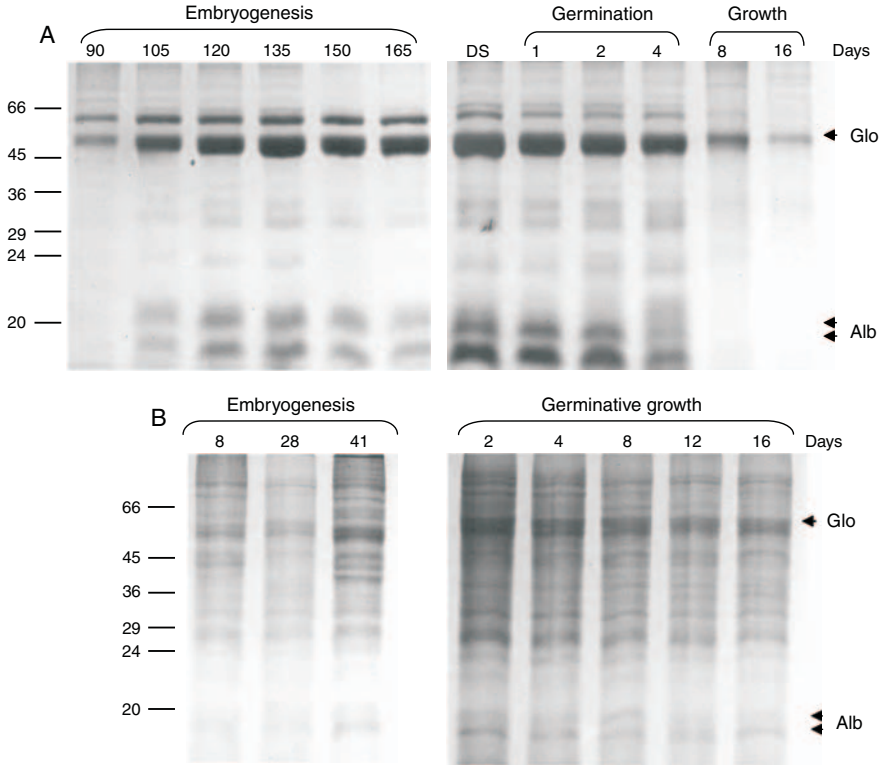

Figure 2. Analysis of electrophoretic patterns of proteins in zygotic and somatic oil palm (*Elaeis guineensis*) embryos by SDS-PAGE. (A) Zygotic embryos harvested during embryogenesis (90–165 days post anthesis), from dry seed (DS) and during germinative growth (after 1–16 days of in vitro germination). (B) Somatic embryos harvested during embryogenesis (8–41 days after plating) and during germinative growth (after 2–16 days of in vitro germination).

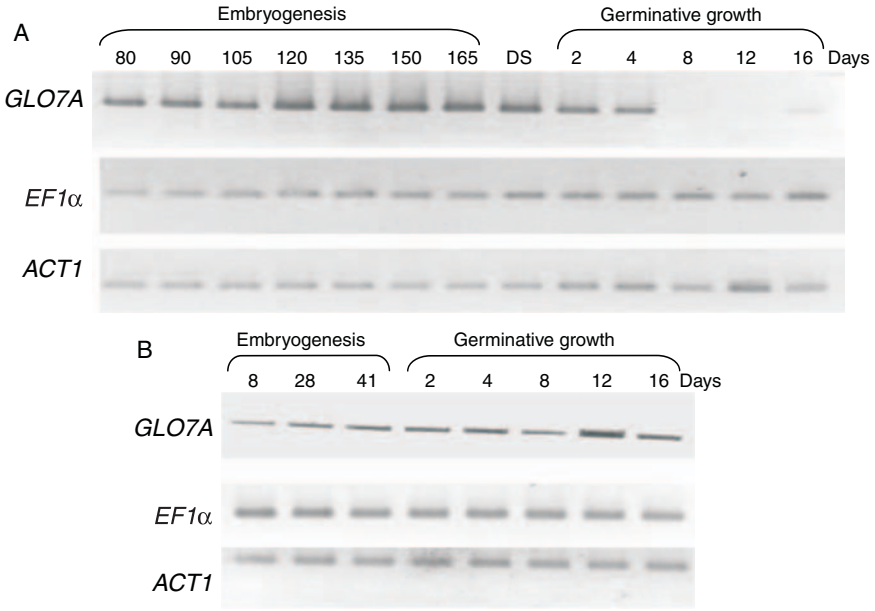

Figure 3. Analysis of expression of *GLO7A* genes in zygotic and somatic oil palm (*Elaeis guineensis*) embryos. Electrophoregram of RT-PCR products using total RNA from (A) zygotic embryos harvested during embryogenesis (90–165 days after pollination), from dry seed (DS) and during germinative growth (after 1–16 days of in vitro germination), and (B) somatic embryos harvested during embryogenesis (8, 28 and 41 days after plating) and during germinative growth (after 2, 4, 8, 12 and 16 days of in vitro germination). Oil palm *EF1*α and *ACT1* primers were used as controls.

Characterization of protease activities

Protease active bands with estimated molecular masses of 35, 38, 47, 64 and 100 kDa were identified in zygotic and somatic embryos (Figure 4). The four bands of 64, 47, 38 and 35 kDa were assigned to cysteine proteinases. Throughout zygotic embryogenesis, in dry seed and during germination, no protease activity was detected (Figure 5A). Active bands were detected from Day 4 of germinative growth. Band intensities were strongest during germinative growth in embryos harvested at 8 and 12 DAG and decreased at 16 DAG.

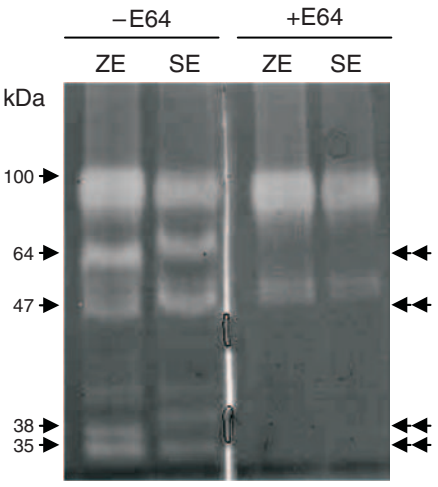

Figure 4. Effect of the cysteine proteinase inhibitor L-*trans*-epoxysuccinyl-leucylamido(4-guanidino) butane (E64) on proteinase activity of extracts from oil palm (*Elaeis guineensis*) zygotic embryos (ZE) harvested during germinative growth (8 days after germination) and from somatic embryos (SE) harvested at Stage III (41 days after plating). In-gel cysteine proteinase activity was detected on SDS polyacrylamide gels copolymerized with gelatine by incubating the gels with (+) and without (–) E64. Double arrows point to the E64-sensitive cysteine proteases.

In somatic embryos, protease active bands were observed at all developmental stages (Figure 5B). From Days 8 to 28 of embryogenesis, similar patterns of active bands with estimated molecular masses of 35, 38 and 100 kDa were observed. Embryos harvested after 41 days of embryogenesis and during germinative growth showed active bands with estimated molecular masses of 64 and 100 kDA. A diffuse signal of active band around 35–38 kDa was also detected. Except for the 47 kDa band, estimated molecular masses of proteinase active bands obtained from zygotic and somatic embryos were similar.

Detection of CPR-like proteins

CPR-like proteins of 35 kD were detected in zygotic and somatic embryos (Figure 6). No signal was detected during zygotic embryogenesis, but a CPR1-like polypeptide was weakly detected in zygotic embryos after 4 days of in vitro germination (Figure 6A). The signal increased during germinative growth (8–16 DAG), concomitantly with increased protease activity (Figure 5A). In contrast, in somatic embryos, a 35 kDa CPR-like polypeptide was detected with a constant activity throughout embryogenesis and germinative growth (Figure 6B).

Identification of cysteine proteinase cDNA clones

Among cDNA clones identified in the oil palm EST database, three clones exhibited strong similarities with cysteine proteinase genes of rice, maize and barley (Rogers et al. 1985, Watanabe et al. 1991, Pechan et al. 1999). They were named *CPRZ*, *CPRF* and *CPRS1* in accordance with the cDNA library from which they were isolated (zygotic embryo, female inflorescence and suspension culture, respectively). The *CPRZ*, *CPRF* and *CPRS1* sequences contained ORFs of 1410, 1410 and 1077 bp and encoded polypeptides of 470, 469 and 358 amino acids, respectively. The deduced amino acid sequences of these cysteine proteinases are presented in Fig-

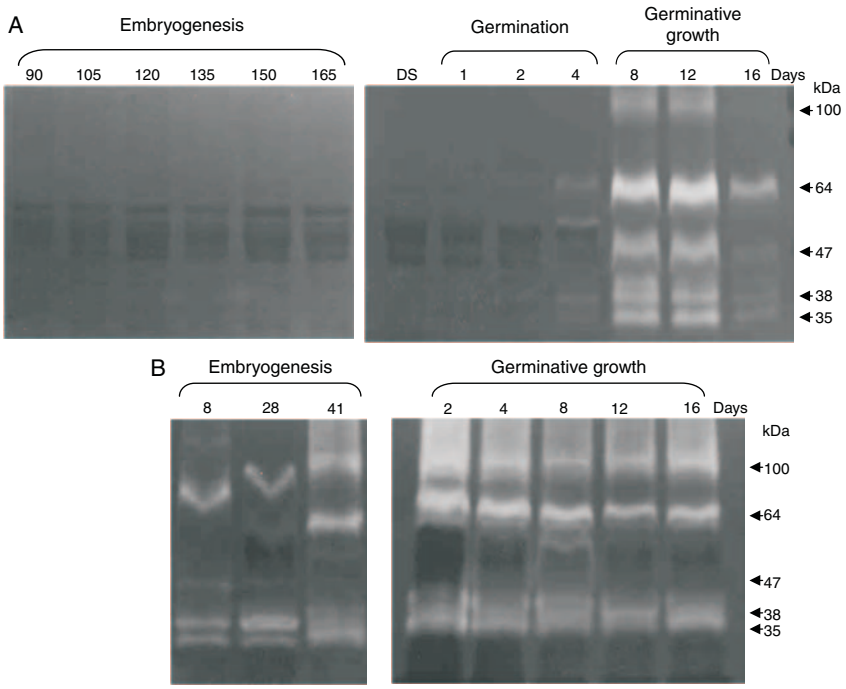

Figure 5. Analysis of electrophoretic patterns of in-gel protease activity during development and germination of zygotic and somatic oil palm (*Elaeis guineensis*) embryos by SDS-PAGE (7.5% polyacrylamide) copolymerized with gelatine. (A) Zygotic embryos harvested during embryogenesis (90–165 days after pollination), from dry seed (DS) and during germinative growth (after 1–16 days of in vitro germination). (B) Somatic embryos harvested during embryogenesis (8–41 days after the plating) and during germinative growth (after 2–16 days of in vitro germination).

ure 7. A high degree of similarity was observed among the CPR proteins, CPRS1 being the most divergent. CPRZ shares 74.6% identity with CPRF, but only 29.6% identity with CPRS1.

The deduced amino acid sequences CPRZ, CPRF and CPRS1 had the general characteristics of cysteine proteinases of the papain family (Figure 7). They contained the consensus sequence ERFNIN motif in the N-terminal propeptide, which is specific to the ERFNIN protease subfamily (Karrer et al. 1993). The oil palm CPR sequences also contained the peptidase C1 domain, which is present in all of the papain group of cysteine proteinases. The catalytic triads Cys165-His301-Asn321, Cys164-His300-Asn320 and Cys164-His304-Asn324 were identified in CPRZ, CPRF and CPRS1, respectively (Figure 7).

CPR gene expression in developing and germinating embryos

In zygotic embryos harvested from 80 to 165 DAP and excised from dry seeds, *CPRS1* transcripts were not detected but weak signals were observed for immature embryos with CPRZ and CPRF primers (Figure 8A). In contrast, from 2 to 16 DAG during both germination and germinative growth, transcripts of all three *CPR* genes were clearly detected. The appearance of *CPR* gene transcripts during zygotic embryo germination thus precedes the detection of protease activities (Figure 5A) and CPR-like polypeptides (Figure 6A).

In developing and germinating somatic embryos, *CPRF* and *CPRS1* transcripts were detected but not those of the *CPRZ* gene (Figure 8B).

Discussion

Accumulation and degradation of storage proteins in zygotic embryos

We studied the accumulation of storage proteins and their subsequent degradation during oil palm zygotic embryogenesis, germination and germinative growth. The embryo developmental phase occurring between 89 and 165 DAP corresponded to mid to late embryogenesis, i.e., maturation. During

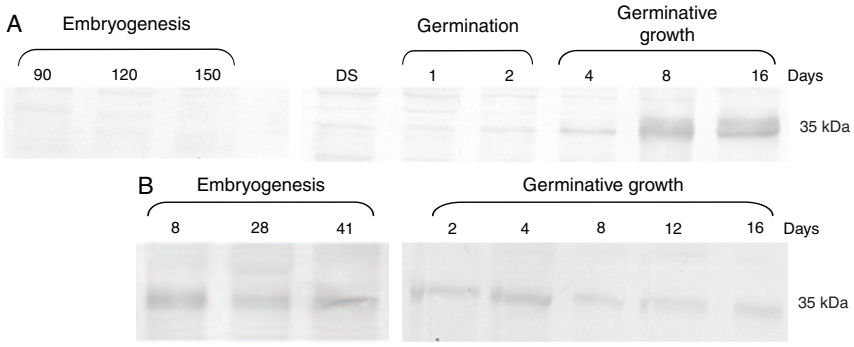

Figure 6. Immunoblot analysis of CPR1-like proteins in oil palm (*Elaeis guineensis*) zygotic and somatic embryos. A 35-kDa polypeptide was detected with a CPR1-specific antibody. (A) Zygotic embryo harvested during embryogenesis (90–150 days after pollination), from dry seed (DS) and during germinative growth (after 1–16 days of in vitro germination. (B) Somatic embryos harvested during embryogenesis (8–41 days after plating) and during germinative growth (after 2–16 days of in vitro germination).

this phase, desiccation tolerance is acquired (Aberlenc-Bertossi et al. 2003) and storage products such as sugars and globulin and albumin proteins are accumulated (Morcillo et al. 1998, Aberlenc-Bertossi et al. 2003). Our results confirm the accumulation of storage proteins and of transcripts of the previously characterized globulin *GLO7A* gene (Morcillo et al. 2001). These results are typical of data observed during late embryogenesis in plants (Galau et al. 1991). Globulins were observed to be mobilized after 4 days of germinative growth and were totally degraded after 8 days.

The mobilization of storage proteins was investigated through the analysis of protease activities and *CPR* gene expression. Based on sequence analyses, several cysteine proteinase genes were identified suggesting that a relatively large multigene family exists in oil palm as in maize and rice (Watanabe et al. 1991, Pechan et al. 1999). During the maturation of zygotic embryos, no protease activities or CPR-like peptides were detected, nor were cysteine proteinase gene transcripts observed to accumulate. The detection of active protease species after 8 days of zygotic embryo germination was concomitant with the start of embryo elongation during the germinative growth phase, the hydrolysis of storage proteins and the detection of CPR1-like proteins.

These results are consistent with events generally occurring during germinative growth (Bewley 1997). The coincidence of globulin disappearance and the increase in protease activities and *CPR* gene expression strongly suggests that cysteine proteinases are involved in globulin mobilization during oil palm zygotic embryo germinative growth. In *Vicia sativa* seeds, the specific degradation of globulin by the papain-like cysteine proteinase, CPR1, CPR2 and proteinase A, was demonstrated by in vitro globulin degradation assays (Fischer et al. 2000). Furthermore, the vacuolar localization of CPR1 and CPR2 in protein bodies of cotyledonary epidermal cells was established by immunocytochemistry (Fischer et al. 2000). To prove the involvement of oil palm CPRs in globulin breakdown according to the criteria proposed by Shutov and Vaintraub (1987), in vitro cleavage of globulins by cysteine proteinases and colocalization of both molecules in the same cellular compartment would need to be demonstrated.

The finding that proteinase activities and gene expression were detected in oil palm zygotic embryos during the germinative growth phase suggests that storage protein mobilization was mainly the result of hydrolysis catalyzed by de

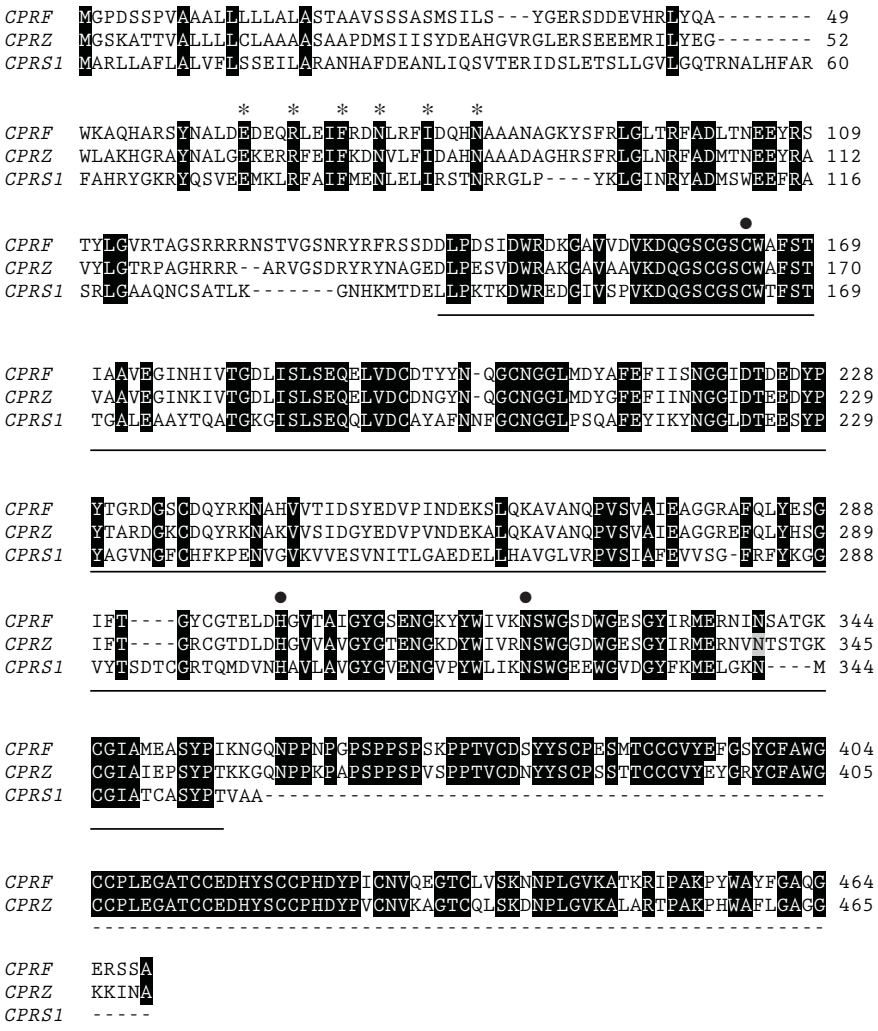

Figure 7. Comparison of the deduced amino acid sequences of the cysteine proteinases encoded by 3 cDNA clones of oil palm (*Elaeis guineensis*). Sequences were designed using ClustalW. The E-R-F-N-I-N motif is marked with asterisks (*). Conserved amino acids of cysteine proteinases are shown in black boxes. The catalytic cysteine-histidine-asparagine triad of cysteine proteinases is indicated by filled circles (●). Peptidase C1 domain of the papain family cysteine proteases is underlined.

novo synthesized proteinases. These results are consistent with those described for other germinating seeds in which degradation of stored protein is catalyzed by de novo formed proteinases (e.g., Ritchie et al. 2000). However, the involvement of stored cysteine proteinases formed during seed development has been demonstrated in the initiation of globulin mobilization in embryonic axes and cotyledons of germinating *Vicia sativa* seeds (Schlereth et al. 2000, Müntz et al. 2001). In rice seeds, transcripts of the cysteine proteinase genes oryzain α and γ were detected specifically during germination, whereas mRNAs of oryzain β were detected in mature dry seeds (Watanabe et al. 1991). As shown for oryzain α and γ (Watanabe et al. 1991), our results suggest that the activities of oil palm CPRs are dependent upon transcript abundance.

Contrasting accumulation and degradation of storage proteins

In oil palm, development and germination of somatic embryos was generally less synchronous than that of zygotic embryos. Furthermore, under our culture conditions, somatic embryos showed continuous development from embryogenesis to germinative growth; they did not undergo the dehydration that follows late zygotic embryogenesis and leads to desiccation, and hence the imbibition phase of germination does not exist in somatic embryogenesis, which is directly followed by germinative growth. A similar developmental sequence was noted for *Cyclamen persicum* Mill. through analysis of mitotic activity (Schmidt et al. 2006). Changes in globulin deposition and gene expression on one hand, and protease activities and *CPR* gene expression on the other hand, revealed that, in oil palm, somatic embryogenesis involves a pattern of developmental regulation distinct from that in zygotic embryogenesis. Globulin gene expression was detected in the somatic embryo during both somatic embryogenesis and germinative growth. In addition, protease activity and CPR1-like peptides associated with the zygotic embryo germinative growth phase were detected during early somatic embryogenesis. A similar situation was observed for glutamine synthase gene expression in maturing pine somatic embryos (Rodriguez et al. 2006). Glutamine synthase *GS1* genes are thought to play a role in the biosynthesis and mobilization of pine seed nitrogen reserves. *GS1a* expression is undetectable in zygotic embryogenesis, whereas it is detected in the cotyledon of somatic embryos along with transcripts of photosynthesis genes and arginase (Rodriguez et al. 2006). Metabolic activities associated with germination thus take place during somatic embryogenesis, showing that these developmental processes are concomitant, in contrast to the developmental sequence in the zygotic system.

We observed less storage protein accumulation in oil palm somatic embryos than in zygotic embryos, as previously reported by Morcillo et al. (2001). Protease activities and CPR-like polypeptides were detected simultaneously throughout somatic embryogenesis and germinative growth. During

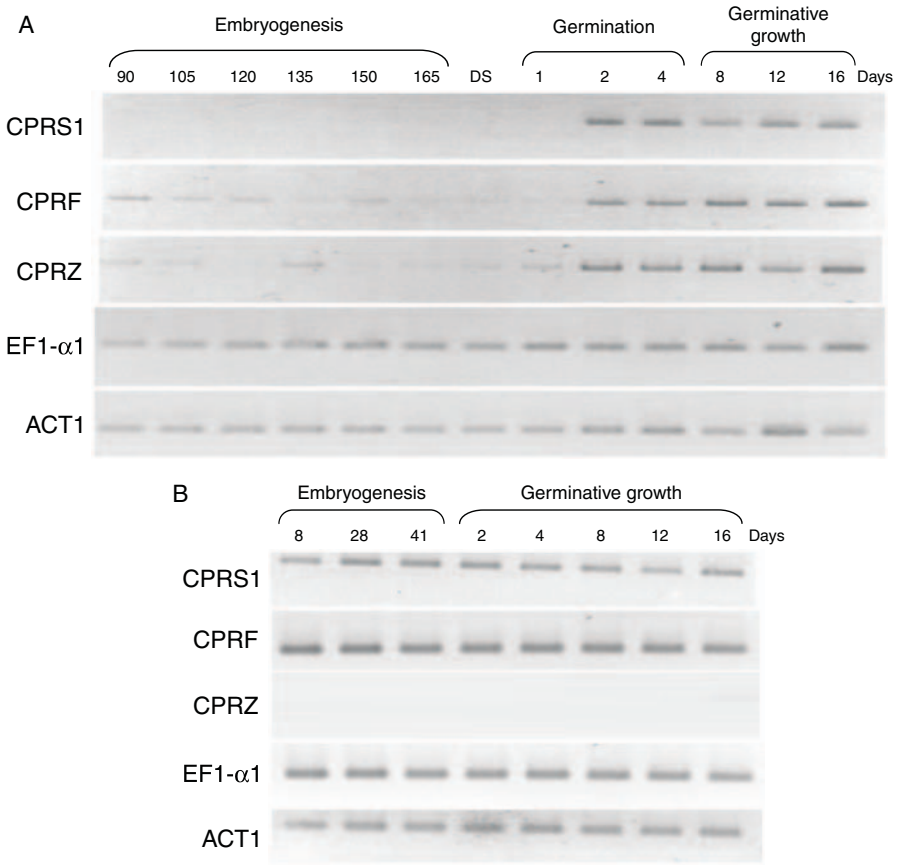

Figure 8. Analysis of the accumulation of *CPR* transcripts in oil palm (*Elaeis guineensis*) zygotic and somatic embryos. Electrophoregram of RT-PCR products using total RNA from (A) zygotic embryos harvested during embryogenesis (80–160 days after pollination), from dry seed (DS) and during germinative growth (after 2–16 days of in vitro germination), and (B) somatic embryos harvested during embryogenesis (8–41 days after plating) and during germinative growth (after 2–16 days of in vitro germination). Oil palm EF1-α1 and ACT1 primers were used as controls.

carrot somatic embryogenesis, active protease bands and cysteine proteinase gene expression were reported and changed in relation to developmental stage (Mitsuhashi et al. 2004). The concomitant low globulin accumulation on one hand and protease biological activity and gene expression on the other hand provide strong evidence for the active synthesis and degradation of globulins during oil palm somatic embryogenesis. Protease-mediated mobilization of storage proteins could thus be causal in their weak accumulation. This assumption is supported by the report of a decrease in the amount of 7S globulin per embryo at the end of oil palm somatic embryo development (Morcillo et al. 1998).

Differential regulation of CPR and GLO gene expression

CPR and *GLO* transcripts were detected in somatic embryos during embryogenesis and germinative growth, respectively, whereas they were not detected at these stages in zygotic embryos. Moreover, transcripts of the *CPRZ* gene expressed in germinating zygotic embryos were not detected in somatic embryos at any developmental stage. Contrasting *CPR* and *GLO* gene expression patterns thus suggest a deregulation of the developmental processes at the transcript level in somatic embryos compared with zygotic embryos. Global gene expression analyses have revealed up- or down-regulation between zygotic and somatic embryogenesis (Che et al. 2006). The lower *GLO7A* RNA amounts may be attributable to alterations in either RNA synthesis or degradation.

In seeds, the temporal regulation of gene expression during plant embryogenesis and germination has been reported in relation to growth regulator balance and environmental constraints (Thomas 1993). The two main signals identified as important in the control of embryo maturation are abscisic acid (ABA) and limited water availability. During the maturation phase, water content declines and ABA, which is the most abundant hormone at this stage, inhibits precocious germination. In contrast, gibberellin exhibits an antagonistic role to that of ABA, stimulating the synthesis of hydrolytic enzymes and mobilization of stored reserves (Jacobsen et al. 1995). Storage protein genes are known to be regulated both temporally and spatially, mainly at the transcriptional level (Bewley et al. 2000), and several studies have highlighted the role of cis- and trans-factors in the regulation of maturation and storage protein gene expression (Vicente-Carbajosa and Carbonero 2005). Cis-regulating elements were identified in promoter regions of *REP-1*, highlighting the transcriptional regulation of this rice cysteine proteinase (Sutoh and Yamauchi 2003). In the oil palm *GLO7A* gene promoter, two motifs resembling ABREs (ABA responsive elements) were identified (Morcillo et al. 2001), suggesting the role of ABA in the regulation of the expression of the gene. In rice and barley seeds, expression of cysteine proteinase genes is induced by gibberellic acid and repressed by ABA (Martinez et al. 2003, Sutoh and Yamauchi 2003). Together, these data highlight the importance of the environment and hormonal status on the regulation of zygotic embryo development and gene expression. An absence, at least in part, of these signals may explain why somatic embryos do not undergo complete maturation. Several parallel studies have indicated that somatic embryo maturation and the prevention of precocious germination can be achieved by manipulation of tissue culture conditions (Merkle et al. 1995, von Arnold et al. 2002). The application of ABA and osmotic stress are key factors in the regulation of these processes. Complete desiccation tolerance in carrot somatic embryos can be induced by an ABA treatment followed by a slow dehydration (Tetteroo et al. 1995). In oil palm, ABA has been shown to stimulate somatic embryo tolerance to rapid desiccation and to repress germination (Aberlenc-Bertossi et al. 2001). Furthermore, amounts of total soluble proteins, 7S globulin content per somatic embryo and *GLO7A* gene expression were all enhanced following ABA and sucrose treatments (Morcillo et al. 2001). These data suggest that somatic embryo maturation can be achieved in vitro, at least partially, but that the exact nature of the regulatory mechanisms that underlie the differences between zygotic and somatic embryogenesis remain to be elucidated.

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