Temporal Association of Ca$^{2+}$-Dependent Protein Kinase with Oil Bodies during Seed Development in Santalum album L.: Its Biochemical Characterization and Significance

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Calcium-dependent protein kinase (CDPK) is expressed in sandalwood (Santalum album L.) seeds under developmental regulation, and it is localized with spherical storage organelles in the endosperm [Anil et al. (2000) Plant Physiol. 122: 1035]. This study identifies these storage organelles as oil bodies. A 55 kDa protein associated with isolated oil bodies, showed Ca$^{2+}$-dependent autophosphorylation and also cross-reacted with anti-soybean CDPK. The CDPK activity detected in the oil body-protein fraction was calmodulin-independent and sensitive to W7 (N-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide) inhibition. Differences in Michaelis Menton kinetics, rate of histone phosphorylation and sensitivity to W7 inhibition between a soluble CDPK from embryos and the oil body-associated CDPK of endosperm suggest that these are tissue-specific isozymes. The association of CDPK with oil bodies of endosperm was found to show a temporal pattern during seed development. CDPK protein and activity, and the in vivo phosphorylation of Ser and Thr residues were detected strongly in the oil bodies of endosperm from maturing seed. Since oil body formation occurs during seed maturation, the observations indicate that CDPK and Ca$^{2+}$ may have a regulatory role during oil accumulation/oil body biogenesis. The detection of CDPK-protein and activity in oil bodies of groundnut, sesame, cotton, sunflower, soybean and safflower suggests the ubiquity of the association of CDPKs with oil bodies.

Keywords: Calcium-signaling — CDPK — Oil bodies — Santalum album L. — Seed development.

Abbreviations: CaM, calmodulin; CDPK, calcium-dependent protein kinase; D, dormant seeds; ER, endoplasmic reticulum; G, germinating seeds; MF, mature fruit; RF, ripe fruit; TAG, triacylglycerol; W7, N-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide.

Introduction

Polysaccharides and lipids act as energy reserves that support various periods of active metabolism in plants (Murphy and Vance 1999, Frandsen et al. 2001). Oil seeds store high levels of lipids (30–75% w/w), especially triacylglycerols, for germination and postgerminative growth of the seedlings (Huang 1985, Tzen et al. 1993, Beisson et al. 2001). Triacylglycerol (TAG) is found in small (0.5–2.5 µm diameter) cytosolic storage organelles called oil bodies that are surrounded by a monolayer of phospholipids embedded with proteins (Frandsen et al. 2001, Tzen et al. 1993, Yatsu and Jacks 1972, Stymne and Stobart 1987, Huang 1996, Chen et al. 1998, Murphy et al. 2001).

Plant oil bodies are remarkably stable and do not aggregate or coalesce either when pressed against each other during seed desiccation, or when isolated by floatation centrifugation (Tzen et al. 1992). Two factors contribute to the stability of oil bodies. One is the electronegative repulsion between oil bodies occurring due to the presence of negatively charged phospholipids on their monolayer (Huang 1992). The second factor is the steric hindrance offered by the abundant structural proteins called oleosins that are present on the surface of oil bodies (Tzen and Huang 1992). Oleosins are 15- to 24-kDa proteins that contain amphipathic N- and C-terminal regions. Their central hydrophobic domain, which is inserted into the TAG matrix, is hypothesized to comprise antiparallel β-sheets connected by a proline knot (Huang 1992).

A few other proteins are associated with oil bodies of diverse plant species (Murphy and Au 1989, Millichip et al. 1996, Tzen et al. 1997, Chen et al. 1998). The sequence of one such minor protein shows that it has an N-terminal calcium-binding domain, and was therefore named calesin (Chen et al. 1999, Naested et al. 2000). Its central hydrophobic domain includes a proline-rich stretch and is believed to interact with the TAG matrix. The C-terminal region of calesin is hydrophilic and has potential phosphorylation sites, three for casein kinase and one for protein tyrosine kinase. Although the function of calesin is yet to be determined, its structural features suggest modulation by Ca$^{2+}$-binding and phosphorylation (Frandsen et al. 2001, Murphy et al. 2001). The identity and biological functions of the other minor oil body-associated proteins remain to be determined.
Although oil bodies are structurally simple, the mechanisms involved in their formation and degradation have remained largely elusive (Murphy et al. 2000, Murphy 2001). As in animals, plant oil bodies are believed to bud from the endoplasmic reticulum (ER) that contains the full complement of the TAG-biosynthetic enzymes (Murphy and Vance 1999, Lacey and Hills 1996). Nonetheless, the signaling events that regulate oil body biogenesis and maturation need to be elucidated. The recent identification of caleosin focuses attention to the probable involvement of calcium during these processes. Ca$^{2+}$ has roles in phospholipid membrane dynamics such as secretion and vesicle fusion (Adolfesen and Littleton 2001, Sudhof and Scheller 2001), and as a second messenger in the regulation of a variety of physiological and metabolic processes in plants (Trewavas 1999, Malho and Trewavas 1996, Sanders et al. 1999, Anil and Sankara Rao 2000, Anil and Sankara Rao 2001a). The downstream events of Ca$^{2+}$-mediated signaling could involve the unique family of Ca$^{2+}$-dependent protein kinases (CDPKs) that are activated by direct Ca$^{2+}$-binding (Harper et al. 1991, Roberts 1993) and are believed to be multifunctional signal integrators in plants (Anil and Sankara Rao 2001a, Harmon et al. 2000).

We have earlier reported the immunolocalization of a CDPK to unidentified spherical organelles in the cells of sandalwood endosperm (Anil et al. 2000). This investigation determines the identity of these storage organelles of Santalum album as oil bodies. More importantly, we present evidence that a CDPK indeed is temporally associated with sandalwood oil bodies, with a concomitant increase in the levels of phospho-Ser/Thr residues in the oil body-associated proteins during seed maturation. The observations suggest involvement of oil body-associated CDPKs and Ca$^{2+}$ regulation during oil body biogenesis.

Results and Discussion

Identification of the spherical storage organelles in sandalwood endosperm as oil bodies

Endosperm is a storage tissue that supplies nutrients to the embryo during germination. In view of this, it was presumed that the spherical organelles that abound sandalwood endosperm (Fig. 1A) are storage bodies. These organelles emitted bright orange fluorescence when stained with Nile blue-A (Fig. 1B) suggesting that they contained liquid neutral fats (Smith 1908, Ostle and Holt 1982). Immunofluorescence analysis of the endosperm sections using anti-oleosin antibodies showed positive staining of the spherical organelles under study (Fig. 1C). A separate experiment wherein sections were subjected to sequential immuno- and Nile blue-staining showed co-localization of Nile blue stain (Fig. 1D) and oleosin antibodies (Fig. 1D') in the spherical organelles (Fig. 1D'') indicating that they are oil bodies. In corroborated, oil-estimation showed that 30% of the dry weight of endosperm was oil. This observation in S. album L. along with the 61% fat content reported earlier in the seeds of Santalum spicatum (R. Br.) A.DC (Flanagan and Barrett 1993) indicates that sandalwood species produce oil-storing seeds. Further, thin layer chromatography of the oil from the endosperm showed a major spot that corresponds to TAG (Fig. 1E), which was absent in lipid extract obtained from shoots, demonstrating the specialization of the endosperm tissue to produce and store TAG. This major spot when isolated and subjected to methanolsysis released fatty acid methyl esters (data not shown), confirming that the oil from endosperm indeed contained the neutral storage lipid, TAG.

The oil bodies were isolated from the endosperm by repeated floatation centrifugation. The isolated oil bodies were perfectly spherical and their diameters ranged from 1 to 3 µm (Fig. 1F). Aggregation of the isolated sandalwood oil bodies occurred when the pH was lowered from 7.2 to 6.5 (Fig. 1G). Aggregation was also observed after 2 h of trypsin treatment, and within 6 h of the treatment, oil bodies coalesced to form larger globules (Fig. 1H). It is well established that seed oil bodies have unique structural stability and do not aggregate or coalesce even when pressed against each other during seed desiccation (Tzen et al. 1993, Tzen et al. 1992). However, isolated oil bodies can aggregate/coalesce under acidic pH condition, which neutralizes their net-surface negative charge, or when subjected to proteolysis, which results in the loss of steric hindrance offered by oleosins (Tzen et al. 1992, Tzen and Huang 1992, Huang 1992). Thus the observation (Fig. 1G, H) clearly shows that the isolated organelles had typical surface properties of plant oil bodies, confirming their identity. The isolated oil bodies positively stained with Nile blue A (Fig. 1I) and anti-oleosin (data not shown). In addition, most of the isolated oil bodies stained with Nile blue A even after being subjected to similar infiltration and deparaffinization processes as the tissue sections (Fig. 1J). This shows that these processes do not completely remove the TAG in the oil bodies authenticating the positive Nile blue staining of the organelles in the endosperm sections (Fig. 1B, D).

Association of CDPK with oil bodies

As reported earlier (Anil et al. 2000) anti-soybean CDPK localized to the spherical organelles, now identified as oil bodies, in sandalwood endosperm (Fig. 1K). Sequential immunolocalization of Nile blue-staining of the endosperm section showed co-localization of Nile blue stain (Fig. 1L) and CDPK antibodies (Fig. 1L') in the oil bodies (Fig. 1L'') indicating that a CDPK was associated with these organelles. This association was further confirmed by the co-localization of Nile blue stain (Fig. 1M) and anti-CDPK (Fig. 1M') on isolated oil bodies (Fig. 1M''). The immunolocalization of CDPK and oleosin was uniform all over the surface of sandalwood oil bodies, both in tissue sections as well as in isolated oil body preparations (Fig. 1). Although, this is in contrast to the halo-like localization that was reported for oleosin expressed in mammalian cells (Hope et al. 2002), the immunostain pattern is similar to that of oleosin and caleosin in rapeseed oil bodies (Naested et al.
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2000). Halo-like localization of oil body surface proteins would be expected ideally, but would depend on thickness of the sections, high magnification and resolution power of the microscope and also on the properties of the antibodies used.

The oil body-associated proteins were analyzed to verify the presence of CDPK. SDS-PAGE analysis of isolated oil bodies revealed a simple protein pattern dominated by a protein of 30 kDa (Fig. 2A). Western blot analysis of the oil body-associated proteins using anti-soybean CDPK showed a cross-reacting protein band at 55 kDa (Fig. 2B). In assays for CDPK activity, the oil body-associated proteins exhibited a higher Ca\(^{2+}\)-dependent substrate phosphorylation activity (7 pmol \(\text{min}^{-1} \text{mg}^{-1}\)) as compared to the total protein extract of endosperm (0.1 pmol \(\text{min}^{-1} \text{mg}^{-1}\)) (Fig. 2C). Thus an enrichment of a CDPK-activity was achieved simply by isolating the oil bodies, further suggesting that a CDPK is associated with these organelles. In addition, a 55 kDa protein was phosphorylated in a Ca\(^{2+}\)-dependent manner when assayed for autophosphorylation activity (Fig. 2D). No protein bands were detected when the oil bodies were analyzed under non-denaturing conditions (data not shown). The proteins in the oil body preparation could be visualized in SDS-containing gels (Fig. 2A), but not in non-denaturing gels (data not shown), indicating that they are associated with the oil bodies and are not soluble con-

**Fig. 1** Identification of the storage organelles in endosperm and the association of CDPK with oil bodies. (A) Bright field image of an endosperm section showing spherical storage organelles. (B) Fluorescence micrograph of an endosperm section stained with Nile blue. (C) Fluorescence micrograph of an endosperm section immunostained with oleosin antibody. (D, D') Fluorescence micrographs of a doubly stained endosperm section showing fluorescence from Nile blue (D) and oleosin antibodies (D'), D and D' are black-and-white representations of the fluorescence micrographs; D'' is the merged image of D and D' showing co-localization in yellow. (E) Thin layer chromatogram of the lipid extracted from endosperm (Endo) showing a spot corresponding to TAG, and from the shoots (Sh) showing no such spot. (F–H) Bright field image of isolated oil bodies (F), isolated oil bodies showing aggregation at pH 6.5 (G), and the large oil globule formed as a result of oil body fusion after 6 h of trypsin treatment (H). (I–J) Fluorescence micrographs of isolated oil bodies either freshly stained with Nile blue (I) or stained with Nile blue following paraffin infiltration and deparaffinization processes (J). (K) An endosperm section immunostained with anti-CDPK. (L, L') A doubly stained endosperm section showing fluorescence from Nile blue (L) and anti-CDPK (L'), L and L' are black-and-white representations of the fluorescence micrographs; L'' is a merged image of L and L'. (M, M') Doubly stained isolated oil body showing fluorescence from Nile blue (M) and anti-CDPK (M'), M and M' are black-and-white representations of the fluorescence micrograph; M'' is the merged image of M and M' showing co-localization in yellow. Scale bar, 5 \(\mu\)m.
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The localization of anti-soybean CDPK to isolated oil bodies (Fig. 1M′) suggests that the enzyme is associated with oil bodies rather than a potential contaminating membrane fraction. A soluble CDPK (swCDPK) from sandalwood embryos has been purified and biochemically characterized (Anil and Sankara Rao 2001b). As this study focuses on a membrane-interacting oil body-associated CDPK from sandalwood endosperm, we felt the need to comparatively analyze these CDPKs found in the two tissue types of sandalwood seeds. These two tissue-specific enzymes showed differences in their rates of histone phosphorylation (Fig. 2E). From the plot of 1/v versus 1/[histone III-S] (figure not shown), the $K_m$ of oil body-associated CDPK for histone III-S was estimated to be 33 µM, as opposed to the $K_m$ of 60 µM exhibited by swCDPK (Anil and Sankara Rao 2001b). In addition, embryo- and oil body associated endosperm-CDPK differed in their sensitivity to W7 inhibition (IC$_{50}$: 6 µM and 23 µM respectively) suggesting that they are tissue-specific isozymes. CDPKs exist as multiple isoforms in a single species, and have been shown to exhibit tissue-specific and developmentally regulated expression (Harmon et al. 2000, Cheng et al. 2002). Variations in enzyme kinetic properties and other biochemical properties imply that members of this family of kinases are designed to respond to various environmental/hormonal signals and to regulate diverse cellular processes.

CDPK was also immunodetected in the oil body-associated protein fraction of other oil seeds such as sunflower, safflower, sesame, groundnut, cotton (Fig. 3A, B) and soybean (data not shown). The oil body-protein fraction of these oil seeds and that of sandalwood endosperm exhibited Ca$^{2+}$-dependent histone phosphorylation activity (Fig. 3C, C′), that was independent of calmodulin (CaM) and sensitive to W7 inhibition (Fig. 3D). Cross-reaction with antibodies raised against the CaM-like domain of soybean CDPK, activation by micromolar Ca$^{2+}$, independence of exogenous CaM and inhibition of activity by the CaM-antagonist W7 are clear indicators for the presence of an endogenous CaM-like domain in the oil-body associated protein kinase. These observations go to suggest that the oil body-associated kinase is indeed a CDPK and also that this association is ubiquitous in plants.

Earlier reports implicate some isoforms of CDPKs in the regulation of starch synthesis or breakdown (Iwata et al. 1998, Fig. 2 Analysis of oil body associated proteins of sandalwood endosperm. (A) Coomassie-blue stained 10% (w/v) SDS-polyacrylamide gel showing the protein profile of the total endosperm protein extract (TP) and that of proteins associated with isolated oil bodies (OB); (B) immunodetection of a 55 kDa CDPK in both TP and OB; (C) phosphorylation of substrate, histone III-S, by TP and OB in the presence and absence of calcium; (D) Ca$^{2+}$-dependent autophosphorylation detected in isolated oil bodies; (E) rate of histone phosphorylation by purified swCDPK (50 ng) from embryos and the oil body CDPK (10 µg of oil body proteins) from endosperm. The percentage activity as a function of time was plotted using the photostimulated luminescence minus background (PSL–BG) emitted by phosphorylated histone III-S. One hundred per cent histone phosphorylation represented a PSL–BG of 800±4.5. Open circles, Oil body CDPK; closed circles, swCDPK. Inset is a representative autoradiogram showing increase in histone phosphorylation with time.

Fig. 3 Detection of CDPK protein and activity in oil bodies of different oilseeds. (A) Coomassie blue stained 10% (w/v) SDS-polyacrylamide gel showing protein profile of isolated oil bodies; (B) immunodetection of cross-reacting proteins of 40–55 kDa with anti-soybean CDPK in the oil body-associated proteins of various oilseeds; (C, C′) oil body-associated Ca$^{2+}$-dependent histone phosphorylation activity in diverse oilseeds. Gray bar, assay carried without CaCl$_2$; Black bars, assay carried out in presence of 0.2 mM CaCl$_2$; (D) effect of calmodulin (CaM) at 200 µM and W7 on the oil body-associated Ca$^{2+}$-dependent histone phosphorylation activity in different oil seeds. Signals in C’ and D represent phosphorylated histone III-S. 1, sunflower; 2, safflower; 3, sesame; 4, groundnut; 5, cotton; S, sandalwood.
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Huber et al. 1996, McMichael et al. 1995). This study is the first to report an association of CDPKs with oil bodies of mature oilseeds, suggesting their possible involvement during oil body biogenesis/degradation.

Implication of CDPK in oil body biogenesis

Isolated oil bodies were obtained from sandalwood endosperm at developmental stages: mature fruit (MF), ripe fruit (RF), dormant seed (D) and germinating seed (G) and not obtained from immature endosperm (stages 1 and 2). The Ca\(^{2+}\)-dependent histone phosphorylation activity associated with the isolated oil bodies of stages MF was strong in comparison to the activity at stage D and was ~10-fold higher than that of stage G (Fig. 4A). Although, CDPK was immunodetected in the total protein extract of endosperm belonging to stages 2, MF, RF, D and G (Fig. 4B), it was detected only in MF and faintly in D in the oil body-protein fraction (Fig. 4C).

Higher in vivo phosphorylation of Ser/Thr residues was immunodetected in oil body proteins of mature and ripe fruit at the 52 and 55 kDa region respectively (Fig. 4D, E). Such strong phosphorylated protein bands were not detected in oil body-protein fraction of stages D and G and also not detected in the total protein of endosperm belonging to all developmental stages (Fig. 4D, E). This suggests increased protein kinase activity during seed maturation and more importantly, that the in vivo substrates that become phosphorylated are oil body associated.

Histological analysis was undertaken to correlate the above observations of temporal protein kinase activity, CDPK accumulation and probable stage-dependent changes in cellular location of CDPK with TAG accumulation, oil body biogenesis and degradation. Endosperm sections belonging to different developmental stages were microscopically analyzed. Oil bodies were visualized only in endosperm sections of mature fruit and dormant seed (Fig. 5C, D). Although, distinct oil bodies were not clearly apparent in the endosperm during seed germination, smaller membranous vesicular structures were abundantly present (Fig. 5E, F). It is likely that these vesicular structures are remnants of the oil bodies that are being utilized during seed germination. The sections of endosperm belonging to immature stages 1 and 2 (Fig. 5A, B) did not show positive Nile blue A staining (Fig. 5A′, B′). However, strong staining was detected in endosperm of stages MF, D and G (Fig. 5C′, D′, E′, F′) indicating the presence of TAG in these stages. These observations indicate that oil body biogenesis and accumulation of TAG in sandalwood endosperm take place during fruit maturation.

Double immunostaining using anti-CDPK and anti-phosphoSer did not show any signal in stage 1 of endosperm development (Fig. 6A, A′, A″, Am). Colocalization of CDPK and phosphoSer residues was observed in endomembranes in stage 2 (Fig. 6B, B′, B″, Bm). Colocalization signals for CDPK and phosphoSer residues at the oil bodies was strong in stage MF (Fig. 6C, C′, C″, Cm) and faint in stage D (Fig. 6D, D′, D″, Dm). However, neither CDPK nor phosphoSer residues were localized to oil bodies in the endosperm of germinating seed (Fig. 6E, E′, E″, Em). It is not clear whether CDPK-localization is cytosolic or vesicular in germinating seeds, from the present data (Fig. 6E, E′, E″, Em). The immunofluorescence intensity for CDPK in the entire endosperm cell was strong in stages MF and moderate in stages 2, RF, D and G (Fig. 6F). However, intensity measurements made in the oil body alone showed strong CDPK-immunofluorescence in stage MF, moderate in RF and D (Fig. 6F) and no immunofluorescence signals were detected in stages 1, 2 and G (Fig. 6F). This can be correlated with the increase in Nile blue-fluorescence intensity at stage MF as compared to stage 2 indicating accumulation of TAG during fruit maturation (Fig. 6F). Oil bodies are yet to develop at stage 1 and 2, and the endomembrane showing immunolocalization of CDPK in stage 2 (Fig. 6B′) may represent ER from which oil bodies are known to bud off. The non-association of CDPK with oil bodies of germinating seed was not altogether surprising as, in contrast to mature fruit, the oil body-protein fraction of stage G shows low Ca\(^{2+}\)-dependent autophosphorylation (data not shown) and substrate phosphorylation activity (Fig. 4A). Further, oil body-
associated CDPK was not immunodetected in Western blots in this stage (Fig. 4C). These observations further implicate that although CDPK is present in stage G, it is not associated with the oil bodies during germination. Such a transient enzyme association to oil bodies has been reported also for lipases that dock on to oil bodies during seed germination (Huang 1992). The temporal change in sub-cellular localization of CDPK in endosperm could be a result of development-specific dissociation of the CDPK from the oil bodies, probably due to proteolysis, or a result of differential expression of oil body-associated and not-associated CDPK isoforms. Further studies are warranted to decide between these alternatives.

The widespread occurrence of oleosins and their high abundance in oilseeds (10–20% total seed protein) initially led to suggestions that they may be involved in oil body biogenesis. However, recent studies with seeds of some tropical and subtropical plants that lack oleosins but have well-developed oil bodies, implies that oleosins play no part in oil body biogenesis (Leprice et al. 1998). In addition, oleosin accumulates later in development than both $\Delta^9$-stearoyl-acyl carrier protein (Fig. 5). Nile blue fluorescence intensity represents TAG accumulation; CDPK immunofluorescence in the entire cell and in the oil body alone is shown. Scale bar, 15 $\mu$m.

**Fig. 5** Oil body biogenesis and the accumulation of TAG in sandalwood endosperm: A, B, C, D, E, F are bright field images representing different stages of endosperm development; A', B', C', D', E', F' are fluorescence micrographs of Nile blue stained endosperm section representing the respective developmental stage; A, A' stage 1; B, B' stage 2; C, C', represent mature fruit (stage MF); D, D' represent dormant seed (stage D); E, E', F, F' represent germinating seed (stage G). Scale bar, 15 $\mu$m.

**Fig. 6** Temporal localization of CDPK and phospho-serine residues to oil bodies and correlation of CDPK-oil body association with oil body biogenesis: Sections of endosperm were doubly stained with antiCDPK and anti phosSer. Rows represent different developmental stages of sandalwood endosperm. A, A', A'', A'' represent stage 1; B, B', B'', B'' represent stage 2; C, C', C'', C''' represent mature fruit; D, D', D'', D''' represent dormant seed; E, E', E'', E''' represent germinating seed. Column comprising A, B, C, D and E represents bright field image; column comprising A', B', C', D', E' represent the localization of anti-CDPK; column comprising A'', B'', C'', E'' represent immunolocalization using anti-phospho-Serine; A', B', C', C', E'' represent merge of the respective bright field, CDPK immunofluorescence and phosSer-immunofluorescence images; F, fluorescence intensity measurements that shows stage dependent TAG accumulation and changes in intracellular localization of CDPK in endosperm. Nile blue fluorescence intensity represents TAG accumulation; CDPK immunofluorescence in the entire cell and in the oil body alone is shown. Scale bar, 15 $\mu$m.
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Fig. 6
that fusion of nascent microlipid bodies after their release from nascent oil bodies during their budding from ER with no functional consequence in oil bodies cannot be ruled out. Some observations made in this study that suggest otherwise are: (a) there is significant increase in CDPK levels in oil bodies of mature endosperm than in the endomembranes of immature endosperm cells (Fig. 6B, C, F); (b) there is an increase in the level of CDPK protein in total protein extract of mature endosperm than that in immature endosperm; (c) there is an increase in the levels of PhosphoSer/Thr residues in oil body-protein fraction in mature seed, suggesting increased protein kinase activity during oil body maturation; and (d) oil body-protein fraction from mature endosperm exhibits strong in vitro Ca\(^{2+}\)-dependent histone and auto-phosphorylation activities. The increased protein kinase activity and strong localization of CDPK to oil bodies in the fruit-maturation stage are data that are consistent with the hypothesis that CDPK has a regulatory role during oil body biogenesis. Nonetheless, these data are correlative and further work is warranted to pinpoint the role of CDPK in oil body biogenesis. The recent identification of the oil body protein, caleosin, that has a Ca\(^{2+}\)-binding site and putative casein kinase phosphorylation sites (Chen et al. 1999, Naested et al. 2000, Murphy et al. 2000) gives support to the hypothesis that calcium and protein kinases regulate these processes in oil seeds. It has been shown that fusion of nascent microlipid bodies after their release from the ER in animal lipid-storing cells is a Ca\(^{2+}\)-dependent process (Murphy and Vance 1999). Nascent oil bodies released from the ER are also believed to undergo several rounds of fusion before becoming mature oil bodies (Frandsen et al. 2001, Huang 1996, Sarmiento et al. 1997). CDPK independently or in conjunction with caleosin (Naested et al. 2000) may act as a regulator of the Ca\(^{2+}\)-dependent fusion of nascent oil bodies. Alternatively, CDPK may be involved in the signaling role of Ca\(^{2+}\) and caleosin in the structural role of Ca\(^{2+}\) in relation to membrane dynamics. Whether their respective roles are interrelated remains to be elucidated.

In conclusion, this paper presents evidence for the development-specific association of some isoforms of CDPKs with plant oil bodies. Oil body structure, biogenesis and breakdown have been of immense interest not only to understand the underlying basic cellular mechanisms but also due to the economic value posed by oil seeds. The higher accumulation and immunolocalization of CDPK, higher in vivo Ser/Thr phosphorylation and stronger Ca\(^{2+}\)-dependent auto- and histone-phosphorylation activities in sandalwood oil bodies during fruit maturation as compared to other developmental stages examined, implicate the involvement of CDPK in oil body biogenesis. These observations pave the way for further study of Ca\(^{2+}\)-mediated regulatory mechanisms operating during oil body biogenesis in plants.

### Materials and Methods

#### Plant material

*Sandelwood* fruits from trees growing on the campus of the Indian Institute of Science were collected at different stages of development viz., 1-week-old fruits (stage 1), 2-week-old fruits (stage 2), mature fruit (MF), ripe fruit (RF) and dormant seeds (D). Germinating seeds (G) were obtained from the greenhouse. Endosperm was scooped out from the fruit/seed after dissection out the embryo. Mature seeds of sunflower, safflower, sesame, groundnut, soybean and cotton were purchased from commercial sources.

#### Chemicals

Histone III-S, W7, CaM, monoclonal anti-phosphoSer and anti-phosphoThr were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Anti-rapeseed oleosin was a gift from Dr. Matthew Hills, John Innes Centre, Norwich Research Park, Norwich, U.K. Horseradish peroxidase-conjugated goat anti-rabbit IgG and horseradish peroxidase-conjugated goat anti-mouse IgG were purchased from Bangalore Genie (Bangalore, India). Radioactive isotope \(\gamma\)-\(\text{ATP}\) was procured from BRIT (Hyderabad/Bombay, India).

#### Tissue sectioning and staining

Endosperm was fixed for 24 h in 70% ethanol, acetic acid and formaldehyde (90 : 5 : 5), upgraded in ethanol series and infiltrated with paraffin wax in \(n\)-butanol at 50°C. Microtome sections of 12 \(\mu\)m thickness were cut and mounted on glass slides. Sections were deparaffinized and rehydrated before staining.

Dewaxed endosperm sections were stained with 1% aqueous solution of Nile blue A for 10 min at 55°C. The sections were washed with milliQ water and with 8% aqueous acetic acid to remove excess stain before fluorescence microscopy using a confocal microscope (TCS MP, single photon imaging system, Leica Microsystems, Wetzlar, Germany). Isolated oil bodies were also stained with Nile blue A after smearing them on to a slide coated with polylysine.

For double immunolocalization, sections were incubated in rinse buffer (1× PBS, 0.05% [v/v] Tween 20) for 15 min and blocked in rinse buffer containing 1% (w/v) BSA, 10% (v/v) NGS (normal goat serum) for 1 h. The slides were placed in a humidified chamber and sections covered with anti-soybean CDPK (Bachmann et al. 1996) and monoclonal anti-phosphoSer (Sigma Chemical Co., U.S.A.) in dilution buffer (1× PBS, 0.05% [v/v] Tween 20, 1% [w/v] BSA and 10% [v/v] NGS) and incubated at 4°C overnight. Following washes in rinse buffer, the sections were incubated for 2 h in FITC-conjugated goat anti-rabbit IgG and TRIC-conjugated goat anti-mouse IgG diluted in dilution buffer (1 : 100). Sections were washed with rinse buffer before mounting in 90% (v/v) glycerol and microscopy.

In some experiments, isolated oil bodies/tissue sections were subjected to immunostaining with anti-oleosin/anti-CDPK prior to Nile blue staining, so as to determine the localization of oleosin/CDPK in relation to the location of TAG in the cells/organelles. Immunofluorescence intensity measurements were carried out with some of the immunolocalization experiments. This was carried out using the confocal microscope and dedicated software that enables fluorescence intensity measurements in any region of interest in the acquired micrograph.

#### Estimation and analysis of oil in the endosperm tissue

Mature endosperm was air-dried, weighed, homogenized and suspended in a 1 : 2 : 1 solution of chloroform, methanol and water and vortexed for 10 min. The organic solvent phase containing the
extracted oil was separated and evaporated to obtain the oil. The extracted oil was weighed and the percentage oil in dry weight of endosperm calculated. As a control, lipids were similarly extracted from sandalwood shoots.

The extracted oil was subjected to thin layer chromatography on silica gel plates using 80 : 20 : 1 of petroleum ether, diethyl ether and acetic acid as the solvent system. The separated lipids were stained with iodine vapor. A spot that showed similar mobility as the standard TAG loaded in the marker lane was scraped out, and extracted with chloroform, methanol (1 : 1). The solvents were evaporated to obtain the isolated lipid. This preparation was dissolved in 4.5 ml of methanol-HCl (5 ml of conc. HCl diluted to 100 ml with methanol) and subjected to methanolysis in a screw-capped tube at 75°C for 1 h and cooled to 4°C for 30 min. The lipids were then extracted with petroleum ether, and separated by thin layer chromatography using the solvent system petroleum ether, diethyl ether and acetic acid in the ratio of 90 : 10 : 1 to examine the formation of fatty acid methyl esters from TAG.

Isolation of oil bodies

Endosperm (10 g) was homogenized in liquid nitrogen using pestle and mortar and suspended in 30 ml ice cold grinding buffer (50 mM Tris-HCl pH 7.2 and 0.5 M NaCl). The homogenate was then filtered through cheese-cloth and centrifuged at 13,650 × g for 30 min at 0°C. The fat pads floating at the top of the centrifuge tubes were carefully scooped out and resuspended in fresh grinding buffer. This floatation centrifugation procedure was repeated thrice with fresh grinding buffer and thrice in milliQ water. Following washes, the fat pads were suspended in 400 µl of cold buffer containing 20 mM Tris, pH 7.2, 2.5 mM EDTA. This constituted the final oil body preparation used for all subsequent experiments. The effect of acidic pH on the stability of the oil body preparation was determined by incubating the preparation for 6 h in 20 mM Tris-HCl (pH 6.5) and 2.5 mM EDTA. The oil body preparation (0.2 ml) was also treated with 0.4 µg of trypsin for 6 h. The effect of acidic pH and trypsin were monitored by light microscopy.

Protein extraction and electrophoresis

Total proteins were extracted from endosperm after homogenizing in extraction buffer containing 1% (v/v) triton X 100 and analyzed by SDS-polyacrylamide gel electrophoresis. Oil body-associated proteins were visualized after boiling the isolated oil bodies in sample buffer and thrice in milliQ water. Following washes, the fat pads were suspended in 400 µl of cold buffer containing 20 mM Tris, pH 7.2, 2.5 mM EDTA. This constituted the final oil body preparation used for all subsequent experiments. The effect of acidic pH on the stability of the oil body preparation was determined by incubating the preparation for 6 h in 20 mM Tris-HCl (pH 6.5) and 2.5 mM EDTA. The oil body preparation (0.2 ml) was also treated with 0.4 µg of trypsin for 6 h. The effect of acidic pH and trypsin were monitored by light microscopy.

Protein kinase assays

Total proteins of endosperm or oil body-associated proteins (10 µg protein) were assayed for calcium-dependent phosphorylation and phosphorylation of the substrate, histone III-S. Assays were carried out in a total volume of 50 µl containing Ca/EGTA buffer (50 mM HEPES, pH 7.2, 10 mM MgCl₂, and 0.25 mM EGTA) with or without 0.2 mM CaCl₂, with (substrate phosphorylation assay) or without (autophosphorylation assay) 1 mg ml⁻¹ histone III-S. The reaction was initiated by adding 10 nM [γ-³²P]ATP (5,000 nCi ml⁻¹). Assay mix for substrate phosphorylation was incubated for 10 min at 30°C and protein phosphorylation monitored either by recording counts on a LKB liquid scintillation counter (Anil et al. 2000) or by resolving the reaction mix by SDS-PAGE and imaging using phosphor imager. Autophosphorylation assays were incubated at room temperature for 20 min and the reaction terminated by boiling in Laemmli’s sample buffer (Laemmli 1970) and resolving by SDS-PAGE. Protein phosphorylation was monitored using the phosphor imager.

Substrate phosphorylation assays were carried out with varying concentrations of W7 and CaM in the assay mixture, both to confirm the CDPK-like activity associated with oil bodies and to determine the enzyme’s IC₅₀ for W7. Oil bodies (10 µg) were also assayed with increasing concentrations of the substrate to determine the oil body-associated CDPK’s Kᵣ for histone III-S. The IC₅₀ for W7 and Kᵣ for histone III-S exhibited by the oil body-associated CDPK from endosperm were compared with those of a purified soluble CDPK from sandalwood embryos (Anil and Sankara Rao 2001b). Time course histone phosphorylation assays were also carried out with 10 µg oil body proteins or 50 ng purified swCDPK from embryos. The assay mix was then analyzed by SDS-PAGE and the extent of histone phosphorylation determined using a phosphor imager.

Western blot analysis

Total endosperm proteins and oil body-associated proteins were resolved on a denaturing polyacrylamide gel and transferred to nitrocellulose membrane following standard conditions (Towbin et al. 1979). The immobilized proteins were then analyzed for cross-reaction with anti-soybean CDPK/anti-phosphoSer/anti-phosphoThr following standard procedures (Anil et al. 2000).

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Reference


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