Calcium-Mediated Signaling during Sandalwood Somatic Embryogenesis. Role for Exogenous Calcium as Second Messenger[1]

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The possible involvement of Ca^{2+}-mediated signaling in the induction/regulation of somatic embryogenesis from pro-embryogenic cells of sandalwood (*Santalum album*) has been investigated. $^{45}Ca^{2+}$-uptake studies and fura-2 fluorescence ratio photometry were used to measure changes in $[Ca^{2+}]_{cyt}$ of pro-embryogenic cells in response to culture conditions conducive for embryo development. Sandalwood pro-embryogenic cell masses (PEMs) are obtained in the callus proliferation medium that contains the auxin 2,4-dichlorophenoxyacetic acid. Subculture of PEMs into the embryo differentiation medium, which lacks 2,4-dichlorophenoxyacetic acid and has higher osmoticum, results in a 4-fold higher $^{45}Ca^{2+}$ incorporation into the symplast. Fura-2 ratiometric analysis corroboratively shows a 10- to 16-fold increase in the $[Ca^{2+}]_{cyt}$ of PEMs, increasing from a resting concentration of 30 to 50 nM to 650 to 800 nM. Chelation of exogenous Ca^{2+} with ethyleneglycol-bis(aminoethyl ether)-N,N'-tetraacetic acid arrests such an elevation in $[Ca^{2+}]_{cyt}$. Exogenous Ca^{2+} when chelated or deprived also arrests embryo development and inhibits the accumulation of a sandalwood Ca^{2+}-dependent protein kinase. However, such culture conditions do not cause cell death as the PEMs continue to proliferate to form larger cell clumps. Culture treatment with N-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide reduced embryogenic frequency by 85%, indicating that blockage of Ca^{2+}-mediated signaling pathway(s) involving sandalwood Ca^{2+}-dependent protein kinase and/or calmodulin causes the inhibition of embryogenesis. The observations presented are evidence to suggest a second messenger role for exogenous Ca^{2+} during sandalwood somatic embryogenesis.

In plants Ca^{2+} ion acts as a second messenger in the signal transduction of a variety of environmental stimuli (Bush, 1995). In analogy to animal systems, stimulus-induced elevations in cytosolic Ca^{2+} are believed to be perceived and transduced by Ca^{2+}-binding proteins and protein kinases (Poovaiah and Reddy, 1993; Trewavas, 1997).

The environmental stimuli and signaling events that trigger and regulate plant embryogenesis are largely unknown. The small size and relative inaccessibility of zygotic embryos in the seeds have contributed to the lack of understanding of this developmental process. Somatic embryogenesis, nevertheless, serves as an alternative experimental system (Zimmerman, 1993). Work on the carrot system has shown that Ca^{2+} enhances embryogenic frequency (Jansen et al., 1990), and its deprivation arrests somatic embryo formation (Overvoorde and Grimes, 1994). These observations are suggestive of an intermediary role for Ca^{2+} during plant embryogenesis.

The role of calmodulin (CaM) as a Ca^{2+} modulator during somatic embryogenesis has also been investigated (Overvoorde and Grimes, 1994). Although, Ca^{2+}-CaM complexes have been localized in the ac-tively dividing regions of carrot somatic embryos, its role as the Ca^{2+} modulator regulating the process of embryogenesis has not been ascertained. Furthermore it is now well established that plants predominantly exhibit a Ca^{2+}-dependent, CaM-independent protein kinase activity. The enzymes responsible for such an activity, namely Ca^{2+}-dependent protein kinases (CDPKs), are encoded by a multigene family and are ubiquitous in plants (Roberts, 1993). CDPKs are unique in having a C-terminal CaM-like domain that can directly bind Ca^{2+}, thereby making CaM unnecessary for their activation (Harper et al., 1991). CDPKs are now implicated to play an intermediary and regulatory role in a variety of developmental and metabolic processes (McMichael et al., 1995; Huber et al., 1996; MacIntosh et al., 1996). We have earlier reported a 55-kD soluble sandalwood (*Santalum album*) Ca^{2+}-dependent protein kinase (swCDPK) that accumulates only in somatic/zygotic embryos, endosperm, and seedlings (Anil et al., 2000). The developmentally regulated, tissue-specific expression of this CDPK isoform in sandalwood suggests its involvement in early developmental processes such as embryogenesis, seed development, and germination.

In the present study we have investigated the role of Ca^{2+} as a second messenger and examined the probable involvement of Ca^{2+}-mediated signaling pathway(s) in the induction/regulation of somatic embryogenesis in sandalwood. Changes in $[Ca^{2+}]_{cyt}$ of pro-embryogenic cell masses (PEMs) were monitored when exposed to culture conditions conducive

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for embryo differentiation. Increase in $[Ca^{2+}]_{cyt}$ was recorded in PEMs upon their transfer from the callus proliferation medium that contains 2,4-dichlorophenoxyacetic acid (2,4-D) to the embryo differentiation medium that lacks 2,4-D and contains higher osmoticum. The study identifies exogenous Ca^{2+} as the source for such an influx and examines the necessity of this Ca^{2+} pool for the process of embryo differentiation. The necessity of exogenous Ca^{2+} pool for the accumulation and activity of swCDPK in embryogenic cultures has also been investigated.

RESULTS

Sandalwood Somatic Embryogenesis

Endosperm of sandalwood dedifferentiated into callus in Murashige and Skoog (MS) medium containing benzylaminopurine and 2,4-D (MS_1). These cells proliferated into a friable embryogenic callus in the presence of 2,4-D alone in the medium (MS_2). The embryogenic callus consisted of two cell types: small, richly cytoplasmic embryogenic cells that aggregate into clumps (PEMs) and the larger elongated nonembryogenic cells containing scanty cytoplasm and large vacuole. MS_3 medium that lacks 2,4-D and contains 2% (w/v) mannitol (differentiation medium)-induced development of somatic embryos from the PEMs. However, under similar growth conditions nonembryogenic cells fail to differentiate into embryos.

Incorporation of $^{45}Ca^{2+}$ and Measurement of Cytosolic Ca^{2+} in the Embryogenic Cells

A 4-fold higher $^{45}Ca^{2+}$ uptake was observed in PEMs that were incubated in MS_3 as compared with those retained in the callus proliferation medium (Fig. 1, A and B). PEMs washed with Triton X-100 overnight showed only 15% of $^{45}Ca^{2+}$ incorporation. This represented cell wall sequestered $^{45}Ca^{2+}$ and has been subtracted from the uptake observed without the detergent wash. Therefore, the assay reflects the $^{45}Ca^{2+}$ incorporated into the symplast.

For the purpose of cytosolic Ca^{2+} measurements, loading of fura-2AM (AM is a commerical notation to denote the ester linkage to fura-2) into PEMs was efficient when incubated for 2 h with 0.02% (v/v) pluronic-127. The Ca^{2+}-bound-dye fluorescence obtained by excitation at 351 nm was negligible in PEMs maintained in callus proliferation medium (data not shown). This fluorescence became intense when the PEMs were perfused with embryo differentiation medium (Fig. 2, A and A'). However, nonembryogenic cells failed to show a similar increase in Ca^{2+}-bound dye fluorescence, upon their transfer to the differentiation medium (Fig. 2, B and B'). Confocal-time-based series of images clearly showed an increase in the Ca^{2+}-bound dye fluorescence upon transfer of PEMs from the callus proliferation medium to the embryo differentiation medium (Fig. 3).
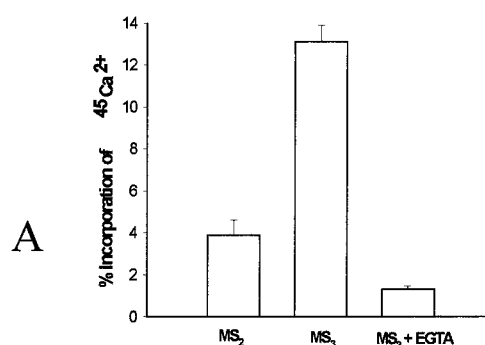

Culture conditions	Incorporation of $^{45}Ca^{2+}$ (ng/250μL p.c.v PEMs)
MS_2	6.3±1.6
MS_3	23.8±2
MS_3+ EGTA	2.1±0.4

Figure 1. Incorporation of $^{45}Ca^{2+}$ into symplast of PEMs. A, Graph represents the percentage incorporation of $^{45}Ca^{2+}$ in callus proliferation medium (MS_2), embryo differentiation medium (MS_3), and in embryo differentiation medium + 1 mM EGTA (MS_3 + EGTA). 100% represents 10,389 cpm obtained from 0.02 μCi of $^{45}Ca^{2+}$ added per reaction. B, Quantity of $^{45}Ca^{2+}$ incorporated into PEMs suspended in MS_2, MS_3, or MS_3 + EGTA. B represents mean values obtained from 10 determinations.

Ratiometric analysis of fura-2 fluorescence (351-/361-nm excitation) from the confocal-images showed increase in the $[Ca^{2+}]_{cyt}$ in PEMs, from a "resting" concentration of 30 to 50 nM in MS_2 to a concentration of 650 to 800 nM ($n = 10$ cells) when perfused with MS_3 medium (Fig. 4A). This influx was not observed when perfused with MS_3 containing 1 mM EGTA (Fig. 4B), thus indicating that Ca^{2+} influx must originate from an exogenous pool and enter the cytosol via the plasma membrane.

Effect of Ca^{2+} Chelation, Ca^{2+}-Channel Blockers, A23187, and N-(6-Aminohexyl)-5-Chloro-1-Naphthalene Sulfonamide Treatments on Embryogenesis

Under optimal differentiation conditions, each PEM gave rise to a cluster of six to eight bipolar embryos. By d 21 of the differentiation cycle, the culture predominantly contained a mixture of bipolar- and torpedo-stage embryos. The dimethyl sulfoxide (DMSO)-treated embryogenic clumps showed no perceivable variation in embryogenic frequency as compared with those developed under optimal differentiation conditions and therefore were considered to exhibit 100% embryogenesis. The percentage embryo-
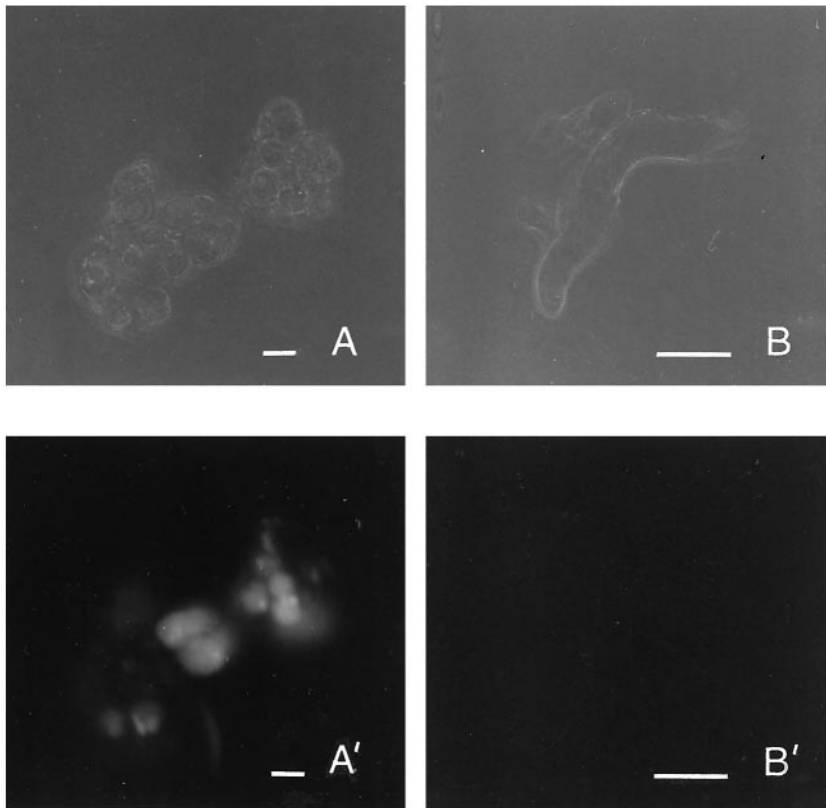
Figure 2. Ca^{2+}-Bound fura-2 fluorescence obtained with excitation wavelength of 351 nm. A, Bright field image of fura-2 loaded pro-embryogenic cells; A', fluorescence image obtained upon transfer of the PEM from MS$_2$ to MS$_3$ medium; B, bright field image of fura loaded nonembryogenic cells; B', fluorescence image after transfer of the nonembryogenic cells from MS$_2$ to MS$_3$ medium. Scale bars = 15 μm.
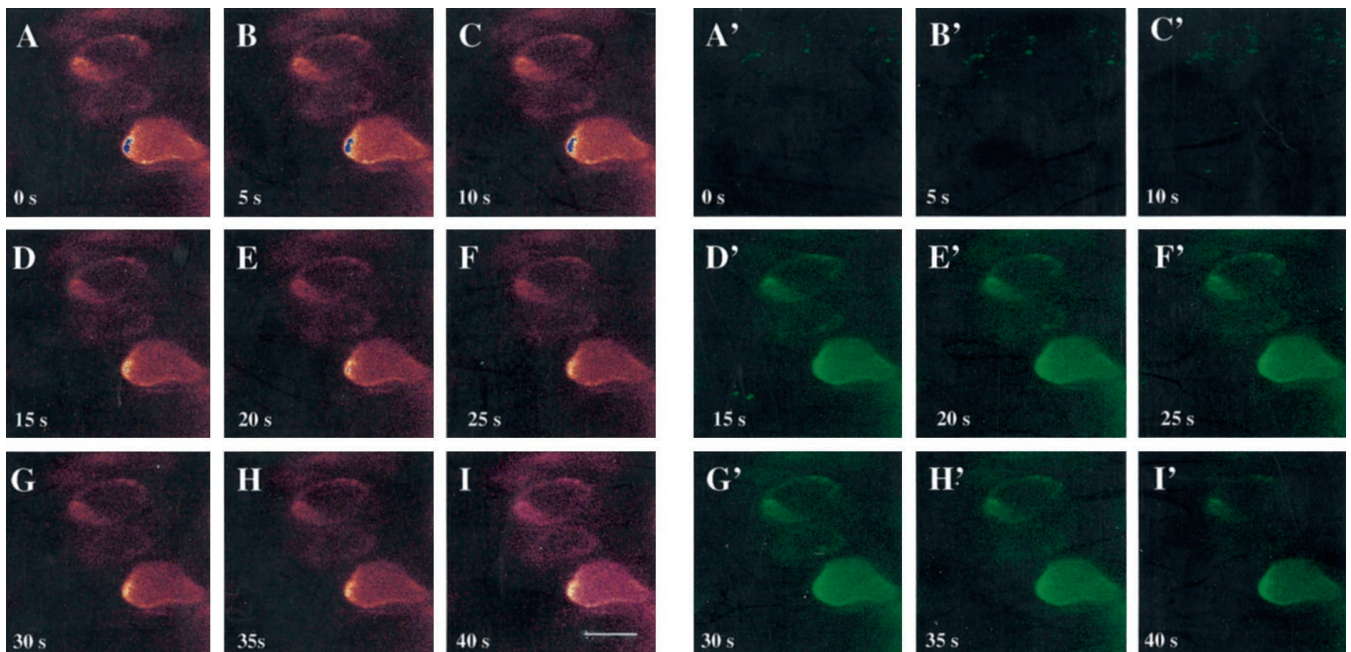

Figure 3. Confocal time-based series of images showing Ca^{2+} homeostasis when fura-2 loaded PEMs in MS$_2$ (MS + 1 mg/L 2,4-D) are perfused with MS$_3$ (MS + 2% [v/v] mannitol). A through I represent the free-dye fluorescence obtained by excitation at 361 nm from 0 time point of perfusion with MS$_3$; A' through I' represent the Ca^{2+}-bound dye fluorescence obtained by excitation at 351 nm at identical time points. Negligible fluorescence in A' through C' indicates the low resting [Ca^{2+}]$_{cyt}$ in pro-embryogenic cells for few seconds after perfusion with MS$_3$ medium. Intense fluorescence in D' represents an influx of Ca^{2+} into the cytosol, which gradually decreases with time (E'–I'). Scale bars = 15 μm.

Figure 4. Ca^{2+} homeostasis in the cytosol of PEMs. Fura-2-loaded pro-embryogenic cells were first perfused with MS_2 (MS + 1 mg/L 2,4-D) to establish the resting $[Ca^{2+}]_{cyt}$. The cells were challenged (arrow) with: A, MS_3 (MS + 2% [w/v] mannitol); and B, MS_3 + 1 mM EGTA. Confocal time-based series of images that depict time-based alterations in Ca^{2+}-bound dye fluorescence and free dye fluorescence in PEMs have been used to determine changes in $[Ca^{2+}]_{cyt}$ by the double wave length ratiometric analysis.
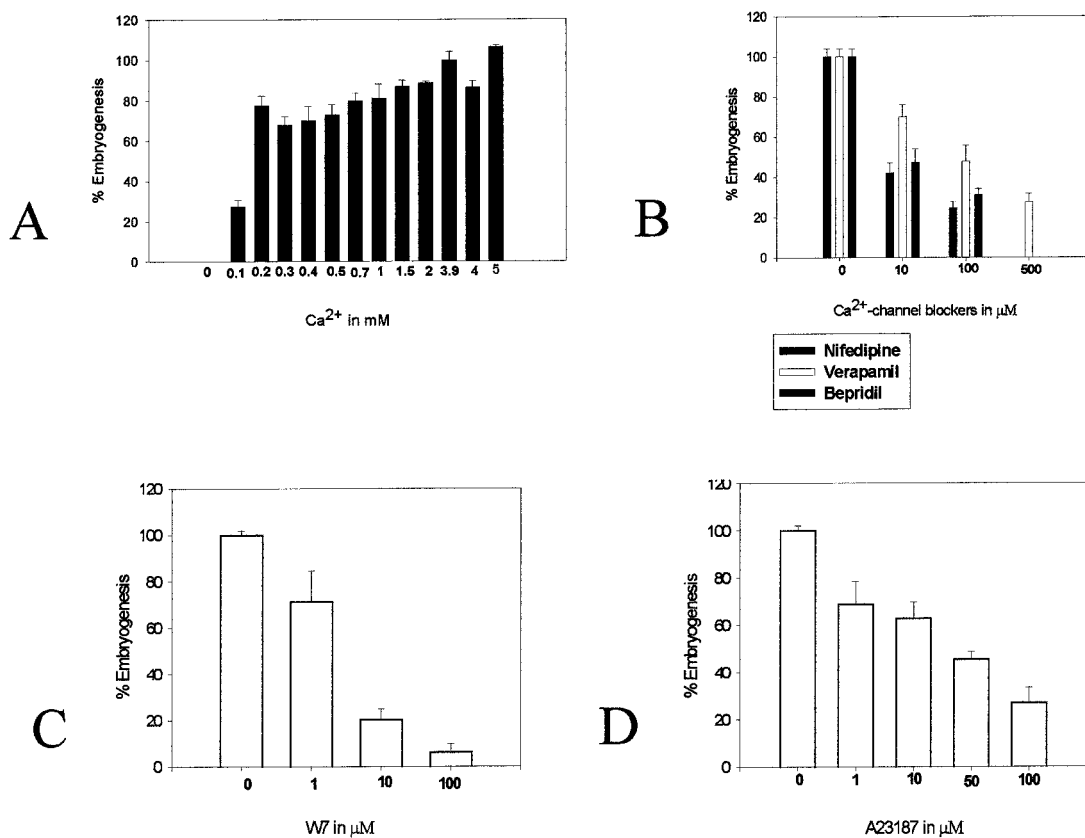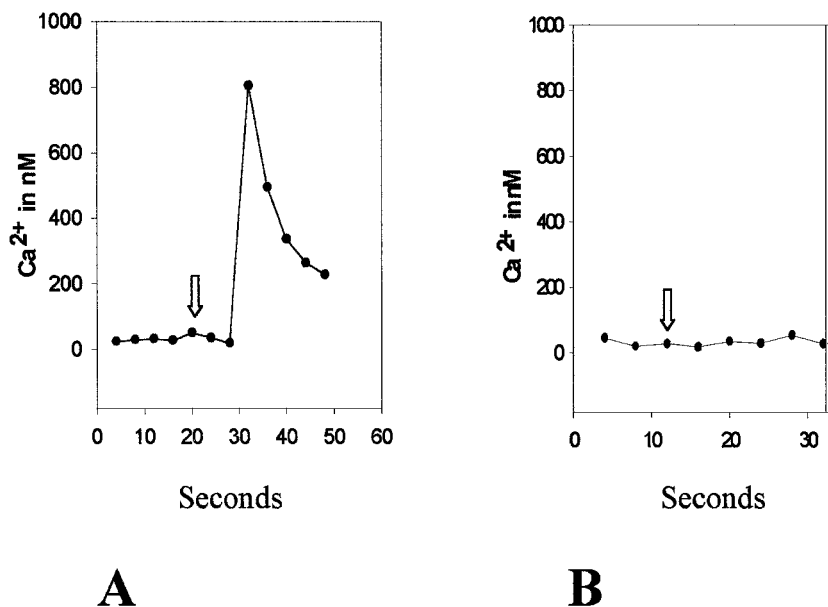
Figure 5. Embryogenic frequency as a function of Ca^{2+}/pharmacological agents in MS_3. A, Percentage embryogenesis as a function of Ca^{2+} concentration in the differentiation medium; 0 mM Ca^{2+} was obtained by the addition of 1 mM EGTA into MS_3 lacking $CaCl_2$. B, Percentage embryogenesis as a function of increasing concentrations of the Ca^{2+}-channel blockers, namely nifedipine, bepridil, and verapamil. C, Percentage embryogenesis as a function of increasing concentrations of W7. D, Percentage embryogenesis obtained with increasing concentration of the Ca^{2+} ionophore A23187. Percentage embryogenesis in the different culture treatments was calculated relative to the 100% embryogenesis obtained in unmodified MS_3 or the DMSO control both containing 3.9 mM Ca^{2+}.
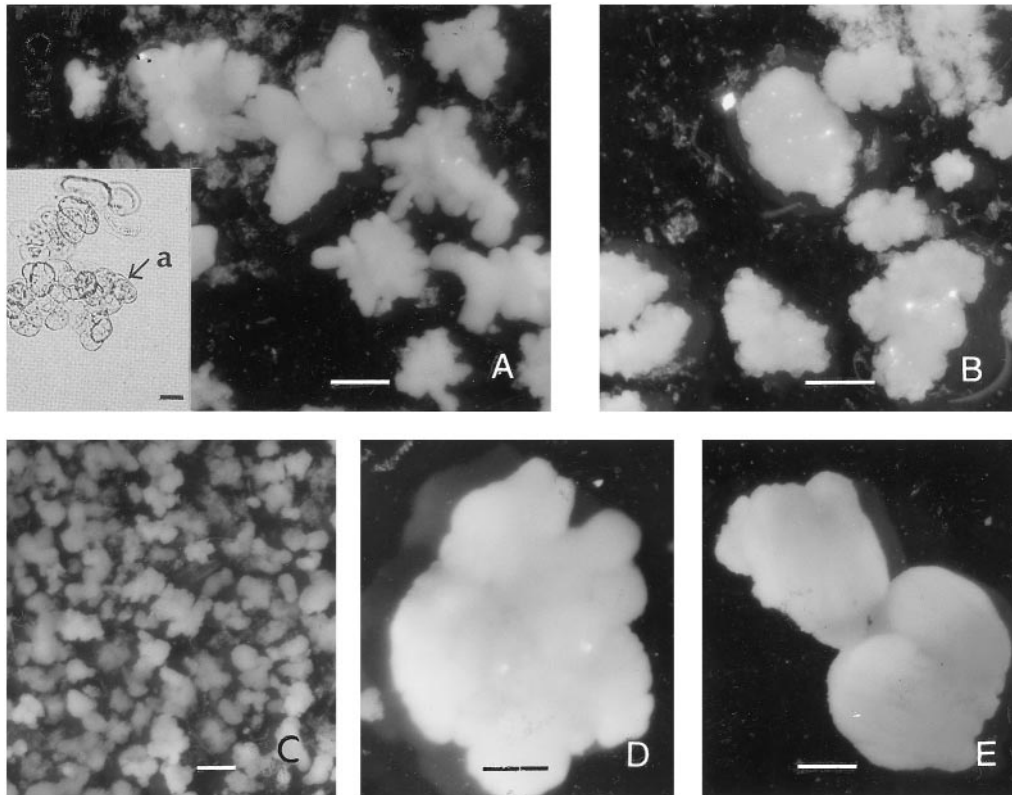
Figure 6. Embryo development under different culture treatments. A, Control showing bipolar- and torpedo-stage embryos emerging from embryogenic cell clumps after d 21 culture incubation; inset a, PEM as seen in the initial inoculum; B, enlarged cell clumps grown in MS_3 medium containing 1 mM EGTA; C, enlarged cell clumps obtained in MS_3 medium containing 500 μM nifedipine; D, fused embryos under Ca^{2+} ionophore treatment; E, enlarged and fused embryos under W7 treatment. Scale bar = 15 μm (inset a); = 6 mm (A–C), = 2 mm (D and E).

genesis obtained with various Ca^{2+} antagonist treatments have been calculated relative to the controls mentioned.

Embryogenesis was completely arrested when all residual Ca^{2+} was chelated from the embryo differentiation medium with the addition of 1 mM EGTA (Fig. 5A). Increasing the concentration of plasma membrane Ca^{2+}-channel blockers, namely bepridil, nifedipine, and verapamil, in the differentiation medium also reduced the frequency of embryogenesis (Fig. 5B), and at concentrations of 500 μM either nifedipine or bepridil, a complete arrest of embryo development was observed (Fig. 5B). Verapamil at 500 μM reduced embryogenesis by 65% compared with the control (Fig. 5B). Differentiation medium containing 100 μM N-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide (W7) or A23187 resulted in 85% and 64% reduction in embryogenesis, respectively (Fig. 5, C and D).

PEMs grew normally and formed bipolar- and torpedo-stage embryos by d 21 of culture incubation in MS_3 medium containing DMSO (Fig. 6A). Although EGTA and Ca^{2+}-channel blocker treatments resulted in arrest of embryo development, the constituent cells of PEMs continued to proliferate to form larger clumps, suggesting that arrest of embry-

ogenesis was not a result of cell death (Fig. 6, B and C). Embryos formed were abnormally large in size and fused in nature in the case of cultures grown under A23187 or W7 treatments (Fig. 6, D and E).

Bipolar embryos grown under optimal conditions showed distinct root and shoot apices and vascular procambial strands (Fig. 7A). The enlarged clumps obtained under Ca^{2+} chelated conditions had normal, compactly arranged cells (Fig. 7B). PEMs of the initial inoculum comprising of 20 to 50 cells per PEM showed a 10- to 100-fold increase in the number of cells by d 21 of culture incubation. No trace of any vascular development was observed in these enlarged cell clumps (Fig. 7B).

Protein Synthesis in Enlarged Embryogenic Clumps Grown in Ca^{2+}-Chelated Differentiation Medium

Many of the soluble proteins that were present in cultures grown under optimal conditions were not detectable in embryogenic cultures grown under Ca^{2+}-deprived/chelated conditions or A23187 culture treatments (Fig. 8A). Nevertheless, L-[³⁵S]Met labeling of proteins in cultures grown under Ca^{2+}-chelated differentiation conditions (MS_3 + 1 mM
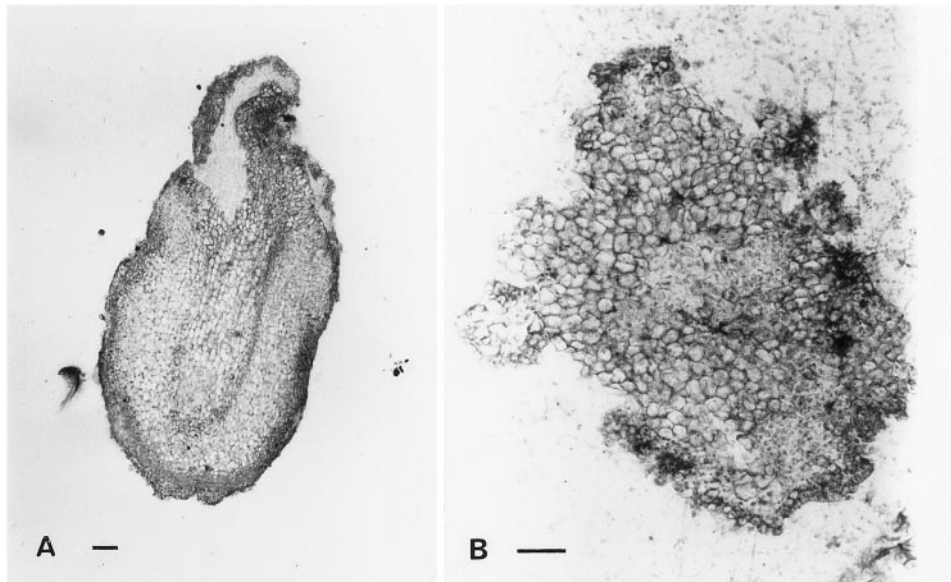
Figure 7. Histological differences in the embryogenic cultures grown in the presence or the absence of Ca^{2+} in MS_3. A, Longitudinal section of torpedo-stage embryo grown under optimal differentiation conditions showing distinct radical and plumular ends and vascular procambial strands. B, Section of an enlarged clump from cultures grown under Ca^{2+}-chelated conditions showing compactly arranged cells but no trace of vascular development. Scale bar = 150 μm.

EGTA) showed that protein synthesis was taking place in the enlarged cell clumps even at d 21 of culture incubation (Fig. 8B).

### swCDPK Accumulation and Activity under Different Culture Treatments

Changes in Ca^{2+}-dependent substrate phosphorylation activity in the soluble protein extracts from different culture treatments are depicted in Figure 9A. Cultures under optimal differentiation conditions showed a 6.8-fold increase in the Ca^{2+}-dependent phosphorylation of the in vitro substrate histone III-S. The W7-culture treatment at a concentration of 100 μM gave moderate Ca^{2+}-dependent histone phosphoryla-

tion activity showing a 5.6-fold increase with addition of Ca^{2+}. However, culture treatment with 1 mM EGTA and 100 μM Ca^{2+} ionophore resulted in low Ca^{2+}-dependent histone phosphorylation activity, increasing only 2.6- and 1.25-fold, respectively, with addition of Ca^{2+}. Cultures treated with 500 μM Ca^{2+}-channel blocker did not exhibit any Ca^{2+}-dependent histone kinase activity (Fig. 9A).

We have identified earlier a 55-kD CDPK in sandalwood embryogenic cultures that exhibited Ca^{2+}-dependent autophosphorylation activity (Anil et al., 2000). W7 treatment of embryogenic cultures did not produce appreciable variation in the autophosphorylation activity of swCDPK relative to control cultures grown under optimal differentiation conditions

Figure 8. Soluble proteins and in vivo protein synthesis under different culture treatments: A, 10% (w/v) SDS-polyacrylamide gel showing Coomassie-stained proteins. Arrows indicate prominent protein bands absent under A23187 treatment and Ca^{2+}-chelated/deprived conditions as compared with the control; B, L-[^{35}S]Met labeling of proteins in embryogenic cultures grown under optimal (control) and under Ca^{2+}-chelated (EGTA) differentiation conditions.
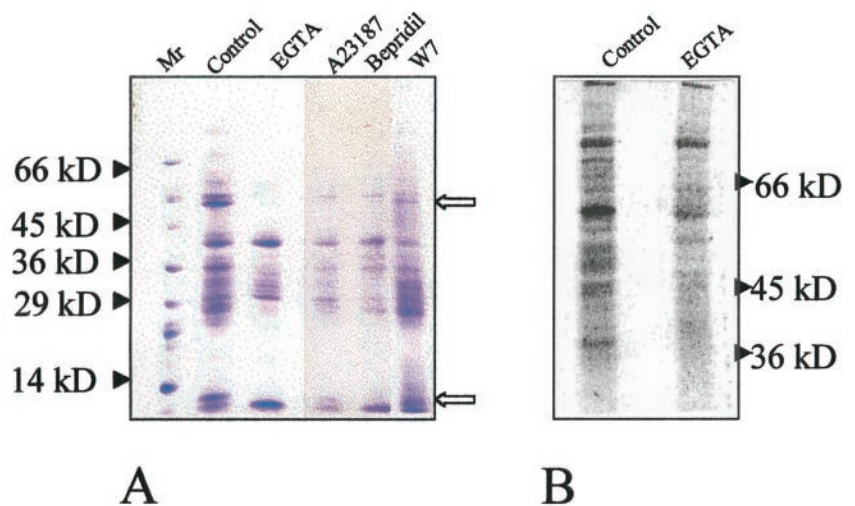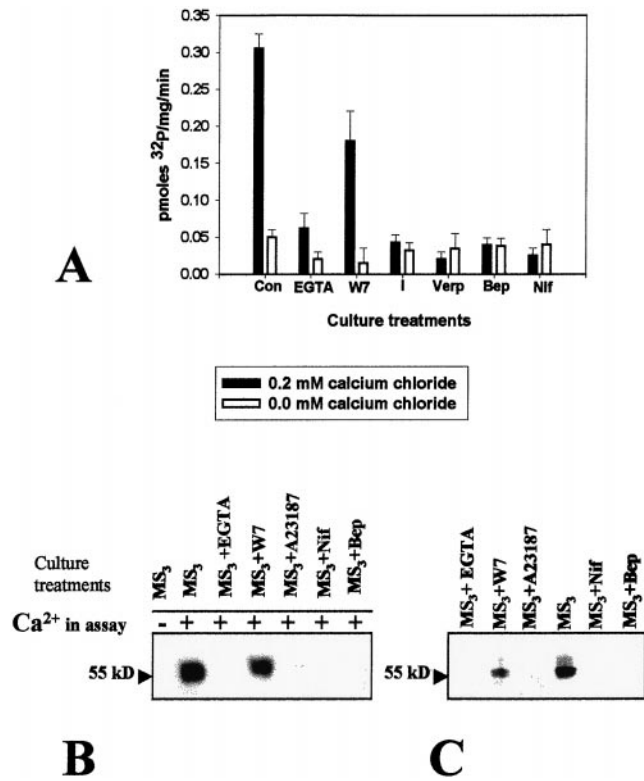
Figure 9. CDPK activity and swCDPK protein in soluble protein extracts of embryogenic cultures subjected to treatments with different pharmacological agents. A, Ca^{2+}-Dependent histone phosphorylation activity in embryogenic cultures grown under optimal differentiation conditions (Con), Ca^{2+}-chelated conditions (EGTA), 100 μM Ca^{2+} ionophore (I), 500 μM nifedipine (Nif), 500 μM verapamil (Verp), and 500 μM bepridil (Bep) treatments. B, Ca^{2+}-Dependent phosphorylation of the 55-kD swCDPK protein determined by assaying in the absence of exogenous substrate followed by SDS-PAGE. C, Immunodetection of the 55-kD swCDPK band using polyclonal antisoybean CDPK in soluble proteins from cultures grown under optimal conditions and different pharmacological treatments.

(Fig. 9B). However, negligible phosphorylation of swCDPK was observed in proteins from cultures subjected to Ca^{2+} chelation or A23187 treatments (Fig. 9B) and the activity was undetectable in those cultures treated with 500 μM Ca^{2+} channel blocker (Fig. 9B). In corroboration the 55-kD swCDPK was strongly immunodetected in the soluble proteins of embryogenic cultures grown under optimal conditions and in cultures treated with W7 (Fig. 9C). It was not immunodetected in cultures grown under Ca^{2+}-chelated, Ca^{2+}-channel blocker, and Ca^{2+}-ionophore culture treatments (Fig. 9C).

DISCUSSION

The data presented in this paper show that the withdrawal of 2,4-D in conjunction with increased osmoticum in the medium can provoke a cytosolic elevation of Ca^{2+} in sandalwood pro-embryogenic cells. As it is the case with several other in vitro

embryogenic systems (Komamine et al., 1992; Sankara Rao, 1996), sandalwood also requires the auxin 2,4-D for tissue dedifferentiation and for subsequent induction of embryogenic-competence in certain cells (PEMs) and withdrawal of 2,4-D for embryo differentiation from the PEMs. Mannitol in the differentiation medium (MS_3) helps simulate the desiccation experienced by seed embryos during maturation and onset of dormancy and facilitates proper embryo maturation, particularly in tree species (Merkle, 1995). Therefore this paper, in essence, provides evidence for cytosolic elevation of Ca^{2+} in pro-embryogenic cells of sandalwood when exposed to culture conditions conducive for embryogenic development.

Although, the $^{45}Ca^{2+}$-uptake studies suggest increased movement of exogenous Ca^{2+} into PEMs in the differentiation medium, it is possible that given conditions for differentiation, PEMs exhibit a nonspecific increase in the uptake of nutrients and ions. However, fura-2-based ratiometric measurements of $[Ca^{2+}]_{cyt}$ confirmed the occurrence of cytosolic Ca^{2+} elevation in the PEMs when exposed to differentiation conditions. The arrest of such an influx by EGTA supports the observation that differentiation conditions induce movement of exogenous Ca^{2+} into the cytosol. Further, the absence of Ca^{2+} elevations in nonembryogenic cells exposed to identical culture conditions indicate the specificity of such a phenomenon to cells predisposed to develop into embryos.

The fold increase in Ca^{2+} uptake caused by transfer of PEMs from MS_2 to MS_3 was lower in determinations obtained with $^{45}Ca^{2+}$-incorporation studies as compared with that with fura-2-based measurement of $[Ca^{2+}]_{cyt}$. This indicates that PEMs in the callus proliferation medium do also take up Ca^{2+} from the exogenous pool. However, the Ca^{2+} that is taken up must rapidly be sequestered into internal organelles so as to maintain the resting $[Ca^{2+}]_{cyt}$ of PEMs in the callus proliferation medium.

To validate the role of exogenous Ca^{2+} as a second messenger in the induction/regulation of somatic embryogenesis, the study investigates the necessity of this Ca^{2+} pool for somatic embryo development per se. The arrest of sandalwood somatic embryogenesis under Ca^{2+}-chelated culture conditions (MS_3 + 1 mM EGTA) suggests that exogenous Ca^{2+} indeed is required for embryogenic development. Concomitantly, the inhibition of embryogenesis by plasma membrane Ca^{2+}-channel blockers confirmed the necessity of this Ca^{2+} pool for the process of embryogenesis. However, it is interesting that PEMs continue to proliferate under such culture conditions, wherein exogenous Ca^{2+} has been chelated or deprived, exhibiting protein synthesis even at d 21 of culture incubation. This implies that although internally stored Ca^{2+} can sustain cell proliferation, it remains an inaccessible store for the process of embryo differentiation.

The Ca^{2+} ionophore A23187 allows diffusion of Ca^{2+} through membranes in an electrically neutral manner, thus neutralizing Ca^{2+} gradients that are normally present across the plasma membrane. Since Ca^{2+} ionophores create an artificial influx of Ca^{2+} into the cytoplasm, they can elicit Ca^{2+}-mediated physiological responses even in the absence of an environmental stimulus (Poovaiah and Reddy, 1993). However, A23187 treatment in our study showed abnormal and reduced embryogenesis. A23187 culture treatment results in PEMs being exposed to extended periods of increased $[Ca^{2+}]_{cyt}$. This could minimize the significance of normal transient Ca^{2+} influxes that occur specifically in response to a stimulus, thus interfering with the process of proper pattern formation and embryo differentiation.

The reduction in the percentage embryogenesis by W7 culture treatment indicates that CaM or a related Ca^{2+}-modulated protein is involved in perceiving the Ca^{2+} signal. This gives strength to the presumption that somatic embryogenesis is regulated by Ca^{2+}-mediated signaling pathway(s). In carrot somatic embryos activated CaM has been localized to regions undergoing rapid cell division, and increase in the level of CaM mRNA observed during globular- and heart-shaped stages (Overvoorde and Grimes, 1994). Yet the modulator role of CaM in regulation of embryogenesis remains unclear. Furthermore, sandalwood embryogenic cultures exhibit a Ca^{2+}-dependent and CaM-independent protein kinase activity and accumulate a developmentally regulated, tissue-specific soluble CDPK (swCDPK) in all stages of embryo development (Anil et al., 2000). This gives credibility to swCDPK as a Ca^{2+} modulator, acting alone or in conjunction with CaM during sandalwood somatic embryogenesis. Although, W7 is a known CaM-antagonist, it also effectively inhibits CDPKs by blocking their C-terminal CaM-like domain (Harmon et al., 1986; DasGuptha, 1994). W7 inhibits the Ca^{2+}-dependent autophosphorylation activity of the 55-kD swCDPK (Anil et al., 2000), and purified swCDPK exhibits the lowest 50% inhibition of initial activity for W7 inhibition among CDPKs characterized so far (V.S. Anil and K.S. Rad, unpublished data). Therefore it can be inferred that reduction in somatic embryogenesis in W7 culture treatments is due to blockage of downstream signaling pathway(s), wherein swCDPK and/or CaM are involved in perceiving the Ca^{2+} signal.

Although the enlarged clumps grown in exogenous Ca^{2+}-chelated/deprived culture conditions exhibit functional protein synthesis machinery, they fail to accumulate detectable levels of swCDPK. This observation further suggests that exogenous Ca^{2+} is also necessary to induce optimal accumulation of swCDPK in the differentiating embryogenic cultures. This is not surprising since Ca^{2+} is known to induce and regulate the expression of CaM and CaM-like proteins that are players in Ca^{2+}-mediated signal trans-

duction. Furthermore the induction of CDPK expression by $CaCl_2$ and its inhibition by EGTA treatments have been reported earlier in mung bean and Sorghum plants (Botella et al., 1996; Pestenacz and Erdei, 1996). The culture treatment with ionophore also causes reduction in the accumulation of swCDPK in embryogenic cultures, suggesting that transitory cytosolic Ca^{2+} influxes may be of significance in the induction of swCDPK expression. The absence or undetectable levels of the enzyme probably contributed to the reduced embryogenic frequency under A23187 culture treatment.

This study thus presents evidence that culture conditions conducive for embryo development elicit a transient movement of exogenous Ca^{2+} into the cytosol of sandalwood pro-embryogenic cells. Culture treatments with Ca^{2+} antagonists further confirm the need for the exogenous Ca^{2+} pool not only for embryogenesis, but also for the expression of swCDPK. Further elucidation of the mechanism of stimulus-response coupling and identification of the different participants in the signaling pathway(s) would provide valuable insights into the process of plant embryogenesis.

MATERIALS AND METHODS

Plant Material

Embryogenic cultures were initiated from the endosperm of sandalwood (*Santalum album*) fruits in MS medium (Murashige and Skoog, 1962) supplemented with 1 mg L^{-1} each of benzylaminopurine and 2,4-D (callus initiation medium-MS$_1$). The endosperm-derived callus was subcultured onto MS medium containing 1 mg L^{-1} 2,4-D (callus proliferation medium-MS$_2$) for proliferation and for embryogenic induction. Cultures were maintained at 26°C ± 2°C under diffuse light conditions of 5 $\mu Em^{-2} s^{-1}$. To isolate PEMs, the callus was suspended in liquid MS$_2$ medium and was strained through meshes of pore size 130, 80, and 50 μm, sequentially. Enriched PEMs were washed twice with the same medium. A packed cell volume (p.c.v.) of 250 μL of the washed cells was resuspended in liquid MS medium lacking 2,4-D and containing 2% (w/v) mannitol (embryo differentiation medium-MS$_3$) for embryo development. The culture was then agitated at 100 rpm on an orbital shaker under diffuse light conditions. Unless mentioned otherwise, 3% (w/v) Suc and 3.9 mM $CaCl_2$ were maintained in the medium.

Chemicals

Bepridil, A23187, W7, histone III-S, DMSO, dry DMSO, and chelex 100 were purchased from Sigma-Aldrich (St. Louis). Nifedipine and verapamil were procured from Boehringer Mannheim (Basel). Polyclonal antisoybean CDPK was a gift from A.C. Harmon (Department of Botany, University of Florida, Gainesville). Horseradish peroxidase-conjugated goat anti-rabbit IgG was purchased

from Bangalore Genie (Bangalore, India). Radioactive isotopes [γ-^{32}P]ATP, [^{35}S]Met, and ^{45}CaCl$_2$ were purchased from BRIT (Hyderabad/Bombay, India).

^{45}Ca^{2+} Uptake by PEMs

Changes in the uptake of ^{45}Ca^{2+} with PEMs either retained in callus proliferation medium (MS$_2$) or subcultured into the differentiation medium (MS$_3$) were determined by first enriching PEMs in liquid MS$_2$ medium, followed by two washes in the same medium lacking Ca^{2+}. Enriched PEMs, 250 μL of p.c.v. each, were subcultured into either liquid MS$_2$ or into liquid MS$_3$ medium, both lacking Ca^{2+}. A control wherein PEMs were inoculated into MS$_3$ containing 1 mM EGTA was also included. Following the addition of 0.02 μCi of ^{45}Ca^{2+}/mL of respective media, PEMs were incubated for 1 h at 26°C ± 2°C under diffuse light conditions. They were then washed twice with 1 mM Na EDTA (pH 8) for 10 min followed by similar washes with 2 mM LaCl$_3$ to remove cell wall-bound Ca^{2+}. The incorporation of ^{45}Ca^{2+} was measured by liquid scintillation counting (LKB, Uppsala). Ten replicates were taken per treatment.

Washes with EDTA and lanthanum do not completely remove cell wall-bound ^{45}Ca^{2+}. To determine the cell wall-sequestered ^{45}Ca^{2+}, the washed PEMs were further treated with 2% (v/v) Triton X-100 overnight.

Measurement of Cytosolic Ca^{2+}

PEMs were enriched in liquid MS$_2$ medium lacking minor nutrients. The loading of the Ca^{2+}-sensitive fluorescent dye fura-2AM was carried out by incubating the PEMs in the above mentioned medium containing 0.02% (v/v) pluronic F-127 and 50 μM fura-2AM at 26°C ± 2°C on a gyratory shaker for 2 h in the dark. The 20% (v/v) stock of pluronic F-127 was prepared in dry DMSO. Following washing of PEMs to remove free fura-2AM, cells were viewed under a fluorescence microscope (Zeiss, Jena, Germany) to monitor loading of fura-2. The dye-loaded PEMs in MS$_2$ medium were made to settle on a coverslip coated with poly-Lys. Cytosolic-free Ca^{2+} was imaged by means of a laser scanning confocal microscope (TCS MP, single photon imaging system, Leica Microsystems, Wetzlar, Germany). Confocal time-based series of images were captured using excitation wavelengths of 351 and 361 nm and by recording emission at 512 nm. The fluorescence intensity in PEMs from the confocal images was determined after subtracting the background fluorescence in each experiment. Ratio (R) of emission from Ca^{2+}-bound fura to emission from free fura (351 nm/361 nm excitation), and the ratios (R_{max} and R_{min}) from a predetermined calibration curve were used to estimate the [Ca^{2+}]$_{cyt}$ on line. The [Ca^{2+}]$_{cyt}$ in PEMs was determined when in MS$_2$ medium and also after perfusion of these PEMs with MS$_3$ or with MS$_3$ containing 1 mM EGTA.

Ca^{2+} calibration was achieved by incubating dye-loaded PEMs in 30 μM A23187 either with 2 mM Ca^{2+} or 5 mM EGTA in the external medium. Ratios of fluorescence in these PEMs represented the highest and lowest [Ca^{2+}]$_{cyt}$,

respectively, and were used in obtaining the calibration curve. The in vivo calibration (Gilroy et al., 1991) used in this study also reflects the responsiveness of the dye to changes in [Ca^{2+}]$_{cyt}$.

Cell Culture Treatments

MS$_3$ containing a range of Ca^{2+} concentrations was prepared to determine the threshold concentration required for embryogenesis. Ca^{2+}-Channel blockers, Ca^{2+} ionophore A23187, and CaM antagonist W7 were dissolved in 2% (v/v) DMSO. MS$_3$ was supplemented with increasing concentrations of one each of these pharmacological agents. Differentiation medium containing DMSO was included in the experiment as a control. Washed embryogenic cell clumps (250 μL of p.c.v.) were inoculated into 20 mL each of the above mentioned media. Each treatment given was in triplicate. Embryo development was monitored by microscopy. Only those PEMs that developed into bipolar-stage embryos were counted after a 21-d culture period.

Tissue Sectioning and Staining

Embryogenic cultures were fixed for 24 h in 70% (v/v) ethanol, acetic acid, and formaldehyde (90:5:5), upgraded in ethanol series, and infiltrated with paraffin wax in butanol at 50°C. Microtome sections of 12-μm thickness were cut and mounted on glass slides, deparaffinated with xylene, and stained with Heidenhain's hematoxylin. The sections were observed and photographed under a microscope (Zeiss).

Extraction of Soluble Proteins

The embryogenic tissue in each of the culture treatments was harvested after 21 d of culture incubation. Tissues were homogenized in liquid nitrogen using a pestle and mortar and suspended in the extraction buffer containing 2.5 mM EDTA, 20 mM Tris [tris(hydroxymethyl)aminomethane]-HCl, pH 7.2, and 1 mM phenylmethylsulfonyl fluoride. The extracts were spun at 13,650g for 30 min at 4°C in a refrigerated centrifuge (Sorvall Products, Newtown, CT). The supernatant containing the soluble proteins was used for further experimentation. Protein concentrations were determined according to the method of Bradford (1976) using bovine serum albumin as the standard. Protein extracts were mixed with Laemmli's sample buffer (Laemmli, 1970), boiled for 2 min, and resolved on a 10% (w/v) SDS polyacrylamide gel.

Protein Synthesis in Vivo

On d 21 of a typical differentiation cycle, 400 μL of p.c.v. of embryogenic cultures was withdrawn from MS$_3$ containing either optimal Ca^{2+} or 1 mM EGTA and incubated for 8 h in the presence of 50 μCi/mL of L-[^{35}S]Met. Labeled samples were centrifuged, and the pelleted embryogenic cultures were washed twice with liquid MS$_3$ to remove free

label. The samples were pelleted again and resuspended in
2.5 mM EDTA, 20 mM Tris-HCl, pH 7.2, and 1 mM phenyl-
methylsulfonyl fluoride. The soluble proteins were ex-
tracted from these samples by sonication using a sonicator
(VibroCell, Sonios and Materials Inc., Danbury, CT)
equipped with a microtip in 20 5-s bursts at a setting of 5.
After separating the cell debris, soluble proteins were re-
solved on an SDS polyacrylamide gel. The gel was treated
with 2% (w/v) sodium salicylate in 30% (v/v) methanol for
30 min, dried, and an image of labeled proteins was ob-
tained using the phosphor imager.

Protein Kinase Assay

Protein kinase activity was determined by measuring the
incorporation of ^{32}P from [γ-^{32}P]ATP into histone III-S. In a
total volume of 0.15 mL the assay mixture contained 1 mg
mL^{-1} histone III-S, Ca/EGTA buffer (50 mM HEPES [4-(2-
hydroxyethyl)-1-piperazineethanesulfonic acid], pH 7.2, 10
mM MgCl$_2$, and 0.25 mM EGTA) with or without 0.2 mM
CaCl$_2$, and 10 μg of the protein sample. The reaction was
initiated with addition of 10 nM [γ-^{32}P]ATP (5,000 nCi/
pmol). The termination of the reaction, spotting of reaction
mixture on glass microfiber filters (type C, Whatman,
Maidstone, UK) and washing of the filters were carried out
according to the method of Putnam-Evans et al. (1990).
Counts were recorded on an LKB liquid scintillation
counter.

Soluble proteins of embryogenic cultures subjected
to various Ca^{2+} antagonist treatments were assayed for
swCDPK autophosphorylation activity. The reaction was
carried out in a total reaction volume of 30 μL, containing
Ca/EGTA buffer with no exogenously added substrate and
incubated for 20 min. The reaction was terminated by ad-
dition of Laemmli's sample buffer (Laemmli, 1970), boiled
for 2 min, and resolved by SDS-PAGE overnight. The gel
was Coomassie-stained to visualize the proteins, dried, and
exposed to x-ray film (Kodak, Rochester, NY) for 24 h.

Immunostaining with Polyclonal Antisoybean CDPK

Protein extracts of embryogenic cultures subjected to
various treatments were resolved by SDS-PAGE and trans-
ferred to nitrocellulose membrane (Towbin et al., 1979).
The blot was blocked in rinse buffer (1× phosphate-
buffered saline, pH 7.4, 0.05% [v/v] Tween 20) containing
1% (w/v) bovine serum albumin for 1 h. This was followed
by incubation in dilution buffer (1× phosphate-buffered
saline, pH 7.4, 0.5% [v/v] Tween 20) containing polyclonal
antibodies directed against the CaM-like domain of soy-
bean CDPK (Bachmann et al., 1996) at a concentration of 15
μg mL^{-1} for 3 h. Excess antibodies were removed by
washing the blot with three changes of rinse buffer for 1 h.
The blot was further incubated with goat anti-rabbit IgG
conjugated to horseradish peroxidase that was diluted to
1:1,000 in dilution buffer. After the removal of non-
specifically bound secondary antibodies, swCDPK was vi-

sualized by incubation in citrate buffer containing diamino
benzidene and hydrogen peroxide in dark.

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