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Virtual screening and *in vitro* evaluation of potential growth regulators against somatic embryogenesis receptor-like kinase (SERK) in *Cocos nucifera* L.

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Coconut (*Cocos nucifera* L.) is one of the most recalcitrant species for *in vitro* regeneration and the efficiency of induction of somatic embryogenesis in coconut explants has remained low. However, with the advent of the genomics era, more information is now available on the involvement of many genes in the induction of somatic embryogenic pathway. Somatic embryogenesis receptor-like kinases (SERKs), belonging to leucine-rich repeat receptor-like kinase super family are reported to play important roles in the process of somatic embryogenesis. In this study, homology based modeling and molecular dynamics (MD) simulation of a coconut SERK protein (CnSERK) was performed for exploring its structural features, functional characterization of its active sites and binding mechanisms of selected plant hormones and growth regulators by docking studies. The 3-D model for coconut SERK was constructed using structure neighbors of the protein in MODELLER and MD simulation was carried out using GROMACS for 5 ns. Fifteen plant growth regulators were docked with the target SERK protein using GLIDE software. An *in vitro* study was then carried out to compare the efficiency of three selected chemicals [adenine sulphate, glutathione and 22(S), 23(S)-homobrassinolide] in enhancing somatic embryogenesis from plumular explants of coconut. Plumular explants were from West Coast Tall cultivar of coconut and were inoculated into Eeuwens Y3 media supplemented with various concentrations of each of the three growth regulators. Among the three growth regulators, glutathione (100 μM) gave the best response for induction of both embryogenic calli and somatic embryogenesis. The results of this study might aid in the development of regeneration protocols for *in vitro* regeneration in coconut

Keywords: Coconut, Docking, Homology modeling, *In vitro* regeneration, Recalcitrant, Tissue culture

Somatic embryogenesis, which forms the basis for cellular totipotency in angiosperms, denotes the process by which somatic cells are developmentally restructured towards the embryogenic pathway[1]. Although somatic embryogenesis has been reported in coconut, a highly recalcitrant crop with respect to *in vitro* regeneration, using juvenile plumular tissues (excised from mature zygotic embryo) as explants, the efficiency of induction of somatic embryogenesis in coconut explants has remained low[2-4]. In order to improve the efficiency of somatic embryo formation, it is important gain a better understanding of somatic embryogenic pathway in this species. Somatic embryogenesis involves differential gene expression and activation of numerous signal transduction pathways. One of the first genes identified in the early stage of somatic embryogenesis was somatic embryogenesis receptor kinase (DcSERK) from *Daucus carota*[5]. Since then, numerous studies have reported that somatic embryogenesis receptor-like kinases (SERKs), belonging to the super family of leucine-rich repeat receptor-like kinases (LRR-RLK), play important roles in the process of somatic embryogenesis[6-10].

Plant receptor-like kinases (RLKs) belong to a class of transmembrane proteins and typically consist of an extracellular receptor domain to perceive a specific signal, a single-pass transmembrane domain to anchor the protein within the membrane and a cytoplasmic kinase domain to transduce the signal

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Abbreviations: BR, brassinosteroid; CASTp, computed atlas of surface topography of proteins; DOPE, discrete optimized protein energy; GLIDE, grid-based ligand docking with energetic; GOR, Garnier-Osguthorpe-Robson; GROMACS, Groningen machine for chemical simulations; LRR, leucine-rich repeat; MD, molecular dynamics; PASS, prediction of activity spectra for substances; PDB, protein data bank; PONDR, predicators of natural disorder regions; RLK, receptor-like kinases; RMSD, root mean square deviation; RMSF, root mean square fluctuation; SAVES, structural analysis and verification server; SERK, somatic embryogenesis receptor-like kinase; SMART, simple modular architecture research tool; SPC, simple point charge (extended); SPP, serine-proline-proline; TDZ, thidiazuron.

downstream via autophosphorylation followed by further phosphorylation of specific substrates. They are mainly involved in perceiving developmental cues and environmental changes and have been implicated in multiple physiological programs ranging from plant development to immunity to microbial infection. The activation of these receptor-like kinases is thought to occur upon ligand-induced complex formation, followed by an activation of downstream-associated proteins. SERK is part of a protein family consisting of five highly conserved LRR-RLK that function in plant signaling pathways[11,12]. SERK proteins are thought to be co-receptors, which are required for transmission of signal[13].

Although success has been achieved in somatic embryogenesis from plumular explants in coconut[4], the number of somatic embryo formation from a single plumule remains low. In order to improve the competence of somatic embryo from a single plumule, it is essential to refine the chemical composition of the media. To attain this goal, it is important to study the role of different growth regulators in induction of somatic embryogenesis. In coconut, a study of SERK expression during somatic embryo induction phase has revealed higher expression of SERK in the embryogenic callus compared to non-embryogenic calli[14].

In this study, we have carried out homology-based modeling of a coconut SERK protein (CnSERK) to establish a basis for its biological role and interaction properties. Molecular interactions with plant growth regulators with CnSERK have also been studied by docking simulations, which could provide mechanistic insight in the role of these chemicals in interacting with SERK. Based on the result obtained through *in silico* studies, an *in vitro* experiment has been conducted by using selected plant growth regulators based on their docking scores in order to enhance the formation of embryogenic calli and somatic embryos from plumular explants of coconut.

Materials and Methods

Data source and structures

The sequence information on SERK protein of *Cocos nucifera* [CnSERK; GenBank: AAV58833.2] was retrieved from the NCBI database (http://www.ncbi.nlm.nih.gov/). Ligand structures of all phytohormones used in this study were retrieved from NCBI PubChem database (http://www.ncbi.nlm.nih.gov/pccompound).

Sequence analysis

Conserved motifs present in CnSERK were analyzed using SMART programme (http://smart. embl-heidelberg.de/). Signal peptide was predicted using SignalP 4.1 server (http://www.cbs.dtu.dk/ services/SignalP/). Transmembrane region was predicted by TMPred Server (http:// www.ch.embnet.org/ software/ TMPRED_form.html). Potential presence of disordered regions within these protein sequences was analyzed using PONDR® VL-XT (www.pondr.com). The positions of phosphorylation sites were analyzed using NetPhos 2.0 (http://www.cbs.dtu.dk/ services/NetPhos/).

Generation of 3-D structure through homology modeling

The sequence of the CnSERK was compared via BLAST (Basic Local Alignment Search Tool)[15] against non-redundant protein sequences database to determine the protein type. BLAST search was performed against Protein Data Bank (PDB) to search for suitable templates. Prior to modeling, template suitability was evaluated by sequence similarity to the target sequence with Blast E-values to ensure the level of sequence identity between target and template must at least 50% which otherwise might result in the deterioration of the model structure. This is an important pre-condition when using multiple templates and needs to be fulfilled for effective modeling. Sequence alignment with templates was performed prior to model construction. Finally, comparative modeling was used to build the three-dimensional model of CnSERK using Modeller9v8 using template amino acid sequences of closest homologues for which X-ray crystal structures are available[16,17.]

Evaluation and validation of the model

Stereochemical analysis and evaluation of the predicted models were carried out by different module in SAVES server (http://nihserver. mbi.ucla.edu/SA-VES) and further loop refinement of each structure, in order to get best build structure with minimum DOPE score, was performed using Modeller. Procheck was used to perform full geometric analysis as well as stereochemical quality of a protein structure by analyzing residue-by-residue geometry and overall structure geometry. Ramachandran plot statistics was used to evaluate the stability of the model. Secondary structure prediction of CnSERK was made by GOR IV (http://npsa-pbil.ibcp.fr/cgibin/npsa_automat.pl? page=npsa_gor4.html). Diagrammatic representations

of the structures were generated using PyMOL 1.3 software (http://www.pymol.org) and RasMol (http://rasmol.org/).

Molecular dynamics (MD) simulations

MD simulations were performed for CnSERK using GROMACS (Groningen Machine for Chemical Simulation, Version 4.5.5 computational package)[18]. The Gromos43a1 force field was applied to the system placed in the cubic box of extended simple charge (SPC) water molecules. The solvated system was neutralized by adding 7 Na^{2+} ions. A constant temperature (300 K) and pressure coupling methods (1 Bar) were employed for maintaining a stable environment in the system. The entire simulation was performed for a time frame of 5 ns.

Virtual screening of plant growth regulators through molecular docking

The structures of all plant growth regulators selected for docking studies were taken from NCBI. Active site residues of the modeled SERK protein were predicted using CASTp server (http://sts-fw.bioengr.uic.edu/castp/calculation.php)[19]. The 3D structure of CnSERK was used for molecular docking with 3D structure of plant growth regulators using GLIDE module of Schrödinger Inc. The receptor grid files were generated using grid-receptor generation program after ensuring that protein and ligands were in correct orientations for docking. The ligands were docked with the modeled CnSERK active site using the "extra precision (XP)" Glide method. The final energy estimation was done with Glide score (G-Score) and a best orientation was taken as the output for a particular ligand. The interactions of the ligands with protein were visualized and the figures were formed using PyMOL 1.3. The PASS server was used to confirm the protein kinase stimulant activity and growth stimulant activity of plant growth regulators (http:// www.pharmaexpert.ru/PASSOnline/)[20].

In vitro studies

The explants used for *in vitro* studies were plumule explants obtained from mature coconut zygotic embryos. The zygotic embryos of 11-month old West Coast Tall (WCT) cultivars were used for the study. The basal medium used was Eeuwens Y3 medium[21] supplemented with 30 g/L sucrose, 1 g/L charcoal and 5.8 g/L agar. It was supplemented with the hormone 2,4-D (74 µM) and varying concentrations of 22(S), 23(S)-homobrassinolide (0.1, 0.5 and 1 µM), adenine sulphate [20 mg/L (135 µM), 30 mg/L (163 µM) and 40 mg/L (217 µM)] and glutathione (100, 200, 400 µM). The pH of the media was adjusted to 5.75 with 1 N NaOH/1 N HCl prior to autoclaving. Y3 medium supplemented with 2, 4-D (74 µM) alone and Y3 with 2,4-D (74 µM) + TDZ (4.5 µM) was used as control.

Embryos were collected from nuts and surface sterilized using sodium hypochlorite. Plumules were extracted from the embryo under sterile condition and inoculated to different media combinations. Each treatment included three replications. Cultures were incubated in dark, where temperature and relative humidity were maintained at 27±10°C and 80 % respectively. After 30 days, the cultures were sub-cultured to media with reduced levels of 2,4-D. Sub-culturing was done at monthly intervals and the observations were collected periodically. After 90 days of culture initiation, the embryogenic structures were transferred to hormone-free media for maturation of somatic embryo. The experiments were replicated thrice with 10 explants per replication. Statistical analysis was performed for both induction of embryogenic calli and somatic embryo formation by using SAS software system.

Results and Discussion

CnSERK structure

A search for CnSERK against the SMART database showed that it encoded a SERK protein with 629 amino acid residues and possessed all the conserved domains present in SERK from other species viz., a signal peptide (residues 1-28), a leucine zipper domain (residues 29-68), five leucine rich repeat (LRR) domains (residues 74-191), the serine-proline-proline (SPP) domain, a transmembrane domain (residues 244-265) and the kinase domain (residues 306-579) at the C-terminal region (Fig. 1). The predicted first 28 amino acids of CnSERK at N-terminal was found to correspond to the signal peptide and the most likely signal peptide cleavage site was between amino acid residues 28 and 29

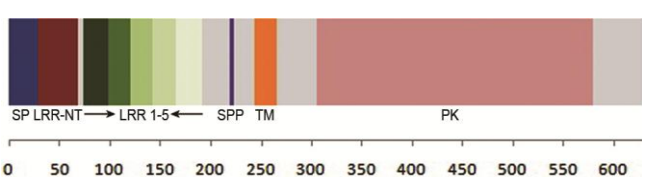

Fig. 1—Domains in CnSERK; SP (N-terminal signal peptide), LRR (leucine-rich repeat), NT (N-terminal), SPP (proline-rich SPP region containing the SPP-motif), TM (transmembrane domain), PK (protein kinase domain) and C (C-terminal domain)

(Fig. 2). TMPred result showed the presence of putative transmembrane helices from position 240 to 264 (Fig. 3).

Although CnSERK was found to be largely structured, there were several short regions of potential disorder as predicted by PONDR®VL-XT. Two main segments of disorder included region 206 to 246 and C-terminal residues from 556 to 629 (Fig. 4). Analysis of phosphorylation sites using NetPhos 2.0 revealed Ser306, Ser397, Ser418, Ser481, Ser485, Ser486, Ser565, Thr340, Thr349, Thr371, Thr373, Thr404, Thr462, Tyr479 and Tyr546 as the potential phosphorylation sites.

Homology modeling of CnSERK

The sequence information on CnSERK (GenBank: AAV58833.2) was collected from the NCBI database. A total of three prospective templates (PDB id: 3TL8_A, 4MN8_B and 4Q3G_A) were selected for modeling based on the search for the best template carried out using BLAST against PDB (Table 1). One of the templates (PDB id: 3TL8_A) with 90% sequence identity against the 335 residues out of 629 (query coverage of 53%) was selected to built the protein kinase domain (residue 305–576) structure of CnSERK. Similarly, two more templates viz., PDB id: 4Q3G_A and 4MN8_B, with 67 % and 72 % sequence identity, respectively were selected to build the LRR regions of CnSERK protein.

The stability of the system was analysed by investigating the RMSD and RMSF properties of the protein simulated for a time frame of 5 ns. It was evident from Fig. 5 that initially the backbone global RMSD fluctuated within the range of 0.2 to 0.25 nm till 4 ns and then reached a stable equilibrium state. Based on RMSF analysis, Gly182, Thr183, Ile184, Pro53, Lys172, Thr52, Thr174, Thr85, Pro84 and Ile66 showed significant fluctuations during MD simulation.

Evaluation of the generated model is an important step for predicting the best model possible. A total of five models of CnSERK were generated and their discrete optimized protein energy (DOPE) scores were calculated using Modeller script. Loop refinement was performed to increase the quality of the model with the best DOPE score. The model 4 (SERK.B99990004.pdb) having the minimum DOPE score (Table 2) was considered as the best model of CnSERK.

Further validation of predicted model by Ramachandran plot of the refined model indicated that out of total 537 non-glycine and non-proline

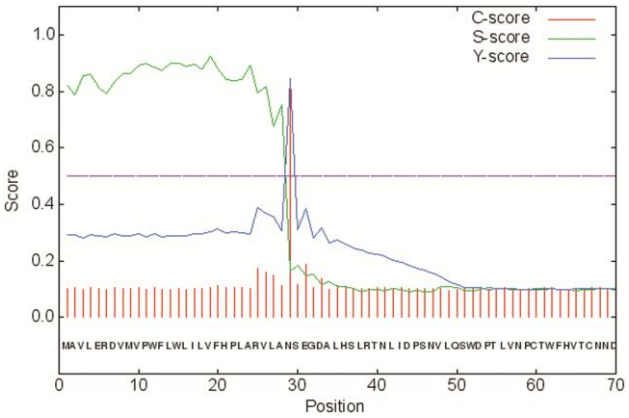

Fig. 2—Prediction of cleavage site of signal peptide of CnSERK according to the progr. SignalP 4.1 [The predicted first 28 amino acids of CnSERK at N-terminal was found to correspond to the signal peptide. The signal peptide cleavage site was observed to be between amino acid residues 28 and 29]

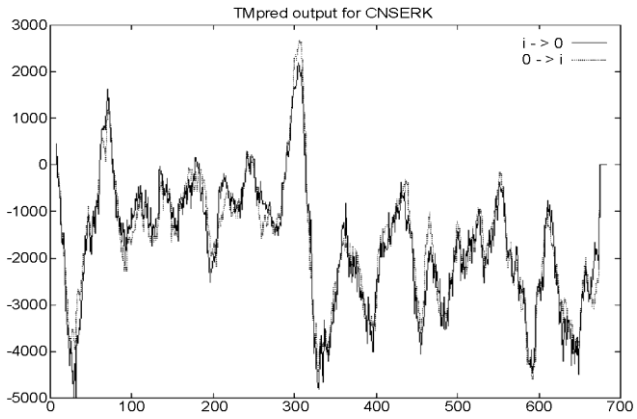

Fig. 3—Predicted transmembrane sequences of CnSERK using TMpredict (TM pred score) [Positive scoring amino acids are likely to be in the membrane and negative scores are hydrophilic]

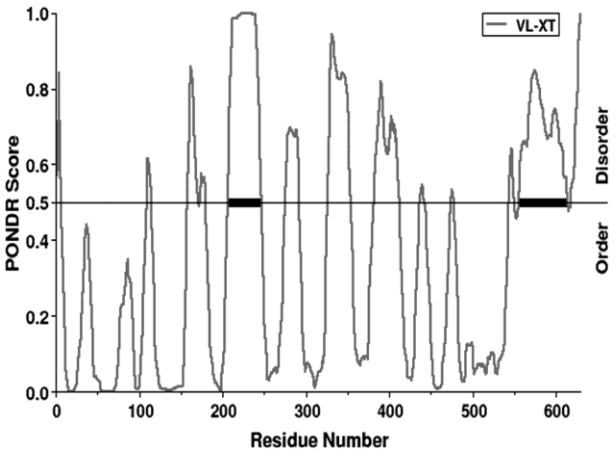

Fig. 4—Predicted disorder in CnSERK [Two main segments of disorder include region 206 to 246 and C-terminal residues from 556 to 629]

Table 1—Selected template structures from BLASTP against CnSERK protein

S. no.	PDB id With Chain	Organism	Title	Sequence identity	E-Value
1	3TL8_A	*Arabidopsis thaliana*	The Avrptob-Bak1 complex	90%	0.0
2	4MN8_B	*Arabidopsis thaliana*	Crystal structure of Flg22 in complex with Fls2 And Bak1 ectodomains	72%	6e-90
3	4Q3G_A	*Arabidopsis thaliana*	Isolated SERK1 co-receptor ectodomain	67%	3e-77

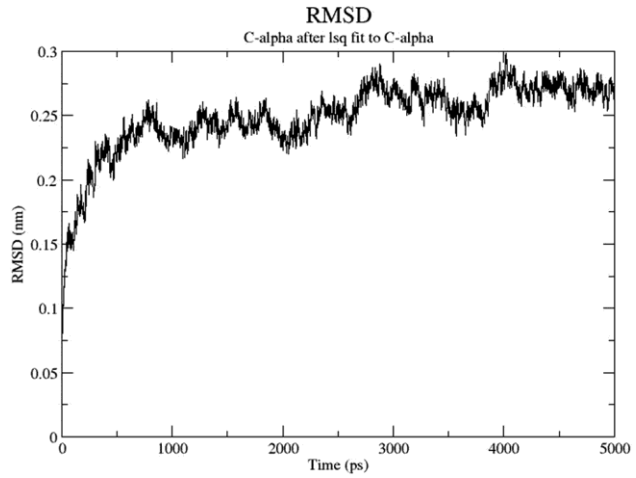

Fig. 5—RMSD graph of CnSERK simulated for a time frame of 5 ns

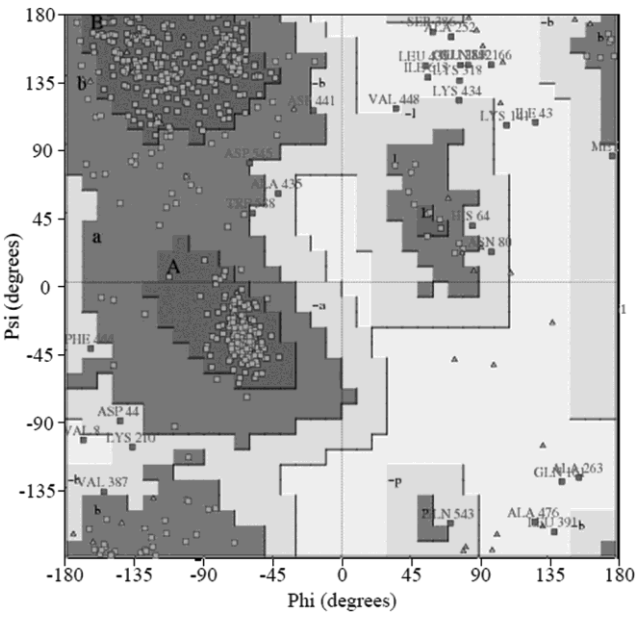

Fig. 6—Ramachandran plot statistics of CnSERK showing most residues (97.7%) in most favoured and additional allowed regions and only 2.3% in the generously allowed and disallowed regions

residues, 525 (97.7%) were in the most favoured and additional allowed regions, 12 (2.3%) were found in the generously allowed and disallowed regions, making this model more acceptable, as compared to other predictable models (Fig. 6).

Table 2—Comparison of DOPE score of models, number of helices, strands and turns in all predicted models and overall quality factor determination through ERRAT

S. no.	Models	DOPE Score	Helices	Strands	Turns
1	SERK.B99990001.pdb	-45019.48	16	17	79
2	SERK.B99990002.pdb	-44622.58	14	16	81
3	SERK.B99990003.pdb	-45251.09	15	12	82
4	SERK.B99990004.pdb	-45980.07	14	24	84
5	SERK.B99990005.pdb	-44652.53	15	18	80

The program GOR IV was used to find the number of helices, strands and turns in CnSERK models. Maximum numbers of strands and turns of model SERK.B99990004.pdb suggested that selected model was more compact and of better quality than other models.

The GOR IV secondary structure prediction revealed the presence of 37.68% α-helices (237 residues), 12.56% extended strands (79 residues) and 49.76% random coils (313 residues) (Fig. 7).

Active site residues of the CnSERK, as predicted by CASTP server were: Pro206, Gly207, Leu311, Gly312, Arg313, Gly314, Gly315, Phe316, Val319, Ala331, Val332, Lys333, Gln346, Glu350, Leu363, Tyr379, Pro380, Tyr381, Met382, Ala383, Asn384, Gly385, Ser386, Ala388, Ser389, Glu393, Pro395, Glu398, Asp432, Lys434, Ala436, Asn437, Leu439, Gly449, Asp450, Phe451, Leu453 and Arg506, which were found on the protein kinase domain of CnSERK, when comparing the position of other cavities present on the given protein (Fig. 8). Finally, the resultant energy minimized CnSERK model (Fig. 9) satisfying all the evaluation criteria was further used for docking analysis with plant growth regulators.

Molecular interactions of ligands with modeled CnSERK

Docking studies of CnSERK protein were undertaken using 15 growth regulators. Adenine sulphate ranked one with the minimum glide score of −5.10 in comparison to other plant growth regulators. Other binding parameter's like vanderwaals interaction energy (−11.92), Coulomb interaction energy (−9.38), Emodel (−28.91) and internal energy (0) of adenine sulphate were found higher than the other growth regulators, suggesting its greater binding affinity, compared to other ligands (Table 3). Two

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         10        20        30        40        50        60        70
          |         |         |         |         |         |         |
MAVLERDVMVPWFLWLILVFHPLARVLANSEGDALHSLRTNLIDPSNVLQSWDPTLVNPCTWFHVTCNND
ccccccccceeceeeeeeeeccccchhhhhccccchhhhhhhccccccceeeeccceeeccceeeeeeccccc
NSVIRVDLGNAQLSGTLVPQLGLLKNLQYLELYSNNISGTIPSDLGNLTNLVSLDLYLNSFTGGIPDTLG
cceeeeeccccccccchhhhhhhhhhhhhhhhcccccccccccccccccchhhhhhhhhcccccccccccc
KLTKLRFLRLNNNSLSGSIPQSLTNITALQVLDLSNNNLSGEVPSTGSFSLFTPISFANNPQLCGPGTTK
hhhhhhhhhhccccccccccccccchhhhhhhhhhcccccccccccccccccccccccceeccccccccccccc
ACPGAPPLSPPPPFISPAPPSSQGSSASSTGAIAGGVAAGAALLFAAPAIGFAWWRRRKPQEHFFDVPAE
ccccccccccccccccccccccccccccchhhhhhhhhhhhhhhhhcchhhhhhhhcccccccccccccc
EDPEVHLGQLKRFSLRELQVATDNFSTKNILGRGGFGKVYKGRLADGSLVAVKRLKEERTPGGELQFQTE
ccchhhhhhhhhhhhhhhhhcccccceeeecccccceeeecccccchhhhhhhhhhcccccccccchhh
VEMISMAVHRNLLRLRGFCMTPTERLLVYPYMANGSVASCLRERPPSEPPLDWTTRRRIALGSARGLSYL
hhhhhhhhhhhhhhhcccccccccceeeecccccccceeecccccccccccccchhhhhhhhhcccceeee
HDHCDPKIIHRDVKAANILLDEEFEAVVGDFGLAKLMDYKDTHVTTAVRGTIGHIAPEYLSTGKSSEKTD
cccccccechhhhhhhhhhhhhhhhhhhhhchhhhhcccccceeeeecccccccccccccccccccccee
VFGYGIMLLELITGQRAFDLARLANDDDVMLLDWVKGLLKEKKLDMLVDPDLQDDYVEAEVESLIQVTLL
eecccchhhhhhhhhhhhhhhhhhhcccchhhhhhhhhhhhhhhchhhhhccccccchhhhhhhhhhhheeee
CTQGSPMERPKMSEVVRMLEGDGLAERWEEWQKVEVVRLDVEMAPPNGNNEWIIDSTDNLHAVELSGPR
ecccccccccccchhhhhhhccccchhhhhhhhhhhhhhhhhhcccccccccceeeeecccceeeeeeecee
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Fig. 7—Secondary structure prediction of CnSERK by GORIV [c: coil, h: helix and e: strand]

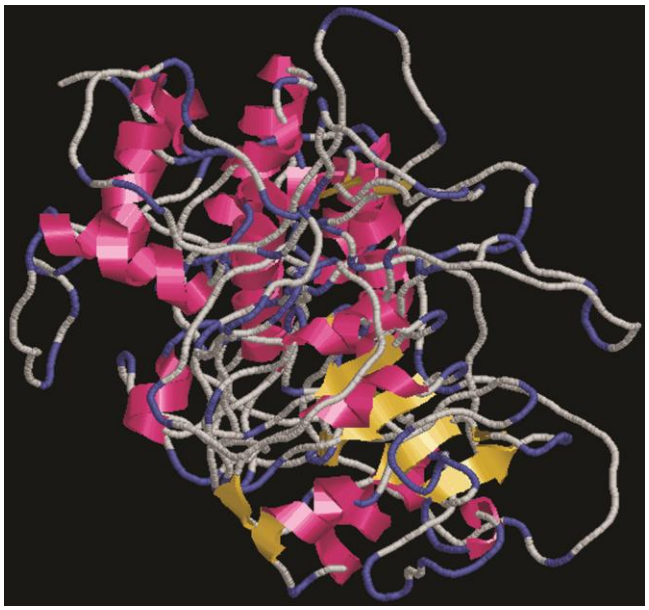

Fig. 8—Active site (green) of SERK protein (surface representation) and blue (stick representation)

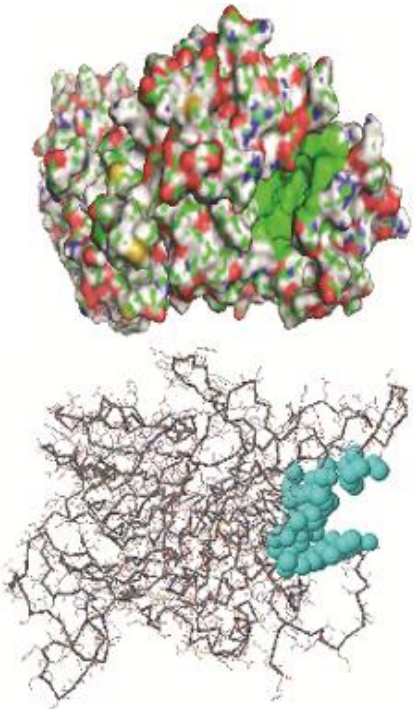

Fig. 9—Refined structure of SERK protein in Rasmol view

hydrogen atoms were found with the length of 1.583 [between the ligand H (14) and protein residues at Glu212] and 2.029 Å [between the ligand H (11) and protein residues at Ala100] (Fig. 10).

In the binding mode, six hydrogen bonds were found between the atoms of the ligand 22(S), 23(S)-homobrassinolide and the amino acid residues of CnSERK. One hydrogen bond of length 1.932 Å was detected between the hydrogen in ligand and oxygen in protein residues Asp144 at a distance of 1.932 Å. There was another hydrogen bond between hydrogen in Gly27 and O (9) in ligand at a distance of 1.921 Å. Two hydrogen bonds were present between the oxygen atoms of protein residue (Phe28 and Gly29) and O (10) in ligand with lengths of 1.965 Å and

Table 3—Results of docking studies between plant growth regulators and coconut SERK protein

Name of the growth regulator	Compound ID	Docking score	Glide G score	Glide H bond	Glide Evdw	Glide Ecoul	Glide Emodel	Glide energy	Glide E internal	Glide posenum	H Bond
Adenine sulfate	9449	-5.074758	-5.100158	-0.45059	-11.9264	-9.38761	-28.9194	-21.314	0	150	2
22(s), 23(s)-Homobrassinolide	11038340	-4.812293	-4.812293	-0.379505	-28.3516	-17.8938	-63.6617	-46.2454	5.310204	221	6
Phenylacetic acid	999	-4.459934	-4.459934	-0.288896	-11.6627	-5.50414	-27.374	-17.1668	0.123403	380	2
1- Naphthalene acetic acid	6862	-4.376173	-4.376173	-0.272008	-13.9125	-4.58073	-28.9541	-18.4932	0.186114	192	1
Glutathione	124886	-4.294762	-4.294762	-0.073417	-18.3312	-15.5148	-49.9911	-33.8459	4.947308	43	4
1,3- Diphenylurea	7595	-4.232029	-4.232029	-0.367352	-17.7006	-7.5557	-32.7731	-25.2563	0.584224	179	1
Indole-3-acetic acid	802	-4.229546	-4.229546	-0.212405	-14.122	-5.03562	-29.5368	-19.1576	0.541509	137	0
Salicylic acid	338	-4.22337	-4.22337	0	-13.8939	-5.67657	-29.3672	-19.5705	1.087062	201	2
2,4,5-Trichlorophenoxy acetic acid	1480	-3.758963	-3.758963	-0.259122	-12.8956	-8.80234	-30.6919	-21.6979	1.607008	262	2
Kinetin	3830	-3.741783	-3.767183	-0.459557	-18.0512	-7.09911	-31.5021	-25.1503	0.853601	30	2
Zeatin	449093	-3.539669	-3.565069	-0.216053	-18.4397	-6.65222	-30.8591	-25.0919	1.479128	230	2
Indole-3-butyric acid	8617	-3.508018	-3.508018	-0.265271	-12.9204	-8.53746	-29.2175	-21.4579	4.143639	180	2
Zeatin	449093	-3.207278	-5.085178	-0.515461	-11.0141	-17.8771	-39.0796	-28.8912	3.517951	197	2
Jasmonate	5281166	-3.162943	-3.162943	-0.369648	-13.7352	-7.43661	-26.4845	-21.1718	6.114143	375	2
Absicic acid	5280896	-3.00297	-3.00297	-0.280458	-17.1941	-5.58534	-27.3288	-22.7794	6.994587	198	2

2.313 Å, respectively. Finally, two hydrogen bonds were present between the atoms (H74 and H75) of ligand and protein residues at Asp212 with length of 1.921 Å and 1.937 Å, respectively (Fig. 10).

Although fifth in terms of docking score, glutathione was selected for evaluation because of its capacity to form four hydrogen bonds with CnSERK. The four hydrogen bonds were present between oxygen in Glu212 and H30 in ligand with 1.992 Å, hydrogen in Gly27 and O6 with 1.728 Å, hydrogen in Lys146 and O7 in ligand with a distance 1.823 Å and hydrogen in Arg181 and O2 in ligand with 2.173 Å (Fig. 10).

An *in vitro* study was conducted by using the three chemicals which gave the least G score. Three concentrations of each chemical *viz*., 22(S), 23(S)-homobrassinolide (0.1, 0.5 and 1 µM), glutathione (100, 200 and 400 µM) and adenine sulphate (135, 163 and 217 µM) were used for the study. PASS server analysis indicated that these chemicals possessed protein kinase stimulant and growth stimulant activities. Out of these, glutathione, an antioxidant was found to be more effective for callus induction and somatic embryogenesis. After 60 days of culture initiation, callus formation was observed to be higher in medium supplemented with 100 µM glutathione with a mean value of 0.96 (Fig. 11). The subsequent transfer of cultures to medium supplemented with reduced 2, 4-D level and then to hormone-free media resulted in the formation of somatic embryos with a mean value of 0.57 (Table 4).

Brassinosteroid treatments confirmed the chemical's encouraging role in callus induction in the least concentration. Among the three concentrations tested (0.1, 0.5 and 1 µM), 0.1 µM 22(S), 23(S)-homobrassinolide, in combination with 2, 4-D, was found to be the better medium for callus induction with a mean value of 0.83 (Fig. 11). The callus formation in the higher concentration (1 µM) was low. There was a decrease in percentage of somatic embryogenesis (0.47) with increase in concentration. Out of the different concentrations of adenine sulphate tested (135, 163 and 217 µM), maximum callusing (0.86) was noticed in plumule cultured in 217 µM concentration (Fig. 11). The somatic embryo induction (0.47) was also observed after fourth sub-culturing of the calli in hormone-free medium. In case of glutathione and 22(S), 23(S)-homobrassinolide treatments, the percentage of induction of embryogenic calli decreased with increase in concentration of chemicals (Table 4).

Overall, Glutathione was found to be the better for somatic embryogenesis after 90 days of culture initiation than the other two chemicals tested. Higher embryo formation (mean value 0.57) was observed in medium supplemented with 100 µM glutathione. In case of adenine sulphate, the rate of somatic embryogenesis increased with increasing concentration of the particular phytohormones (Table 4).

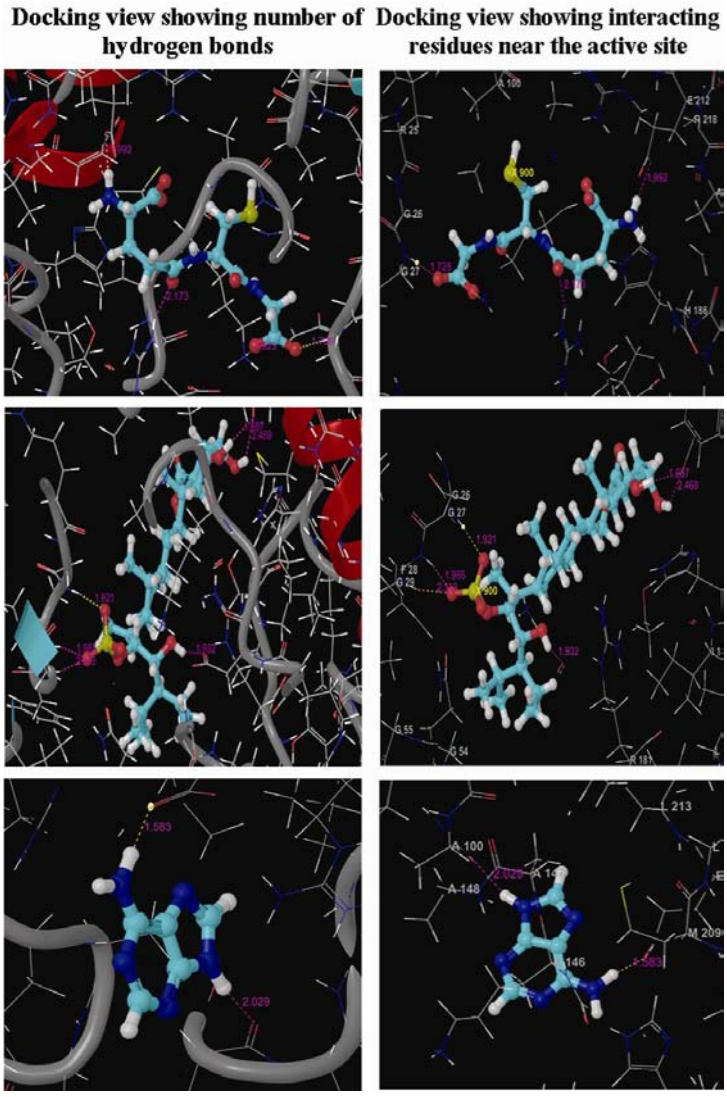

Fig. 10—Docking view showing interaction of CnSERK with ligands

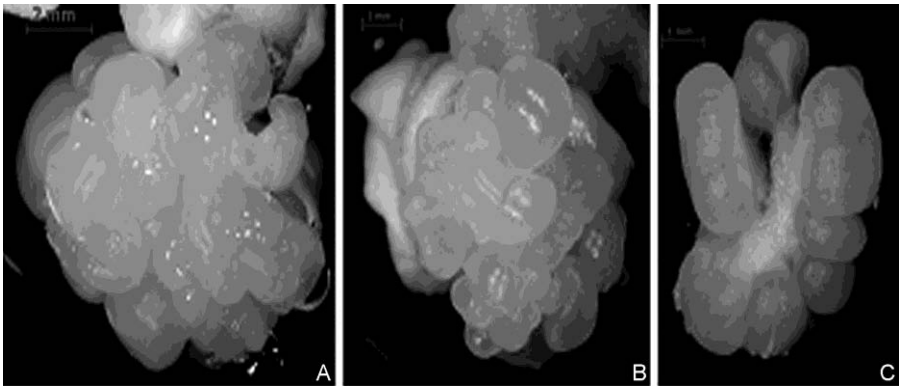

Fig. 11—Somatic embryo formation in coconut plumular explants when cultured in medium supplemented with different growth regulators. (A)Y3 medium supplemented with 2,4 – D (74 µM/L) and Glutathione (100 µM/L) (B) Y3 medium supplemented with 2,4 – D (74 µM/L) and 22(S),23(S)-Homobrassinolide (0.1 µM/L) (C) Y3 medium supplemented with 2,4 – D (74 µM/L) and Adenine sulphate (217 µM/L)

Table 4—Effect of different plant growth regulators on callus induction and somatic embryogenesis from plumular explants of coconut

Chemical	Concentration (μM)	Embryogenic calli (Mean)*	Somatic embryo formation (Mean)*
22(S),23(S)-Homobrassinolide	0.1	0.83 ± 0.37^{abc}	0.47 ± 0.085^{a}
	0.5	0.80 ± 0.40^{abc}	0.43 ± 0.085^{ab}
	1	0.53 ± 0.50^{d}	0.20 ± 0.085^{bcd}
Glutathione	100	0.96 ± 0.18^{a}	0.57 ± 0.085^{a}
	200	0.66 ± 0.47^{bcd}	0.50 ± 0.085^{a}
	400	0.70 ± 0.46^{bcd}	0.37 ± 0.085^{abcd}
Adenine sulphate	135	0.66 ± 0.47^{bcd}	0.13 ± 0.085^{d}
	163	0.47 ± 0.50^{d}	0.19 ± 0.085^{bcd}
	217	0.86 ± 0.37^{ab}	0.47 ± 0.085^{a}
2,4,5-Dichlorophenoxyace-tic acid + Thidiazuron	74 +4.5	0.83 ± 0.37^{abc}	0.40 ± 0.085^{abc}
2,4,5-Dichlorophenoxyacetic acid	74	0.60 ± 0.49^{cd}	0.17 ± 0.085^{cd}

*Means with at least one letter common are not statistically significant

Discussion

One of the bottlenecks in evolving a commercial protocol of *in vitro* propagation in coconut has been low induction of embryogenic calli and somatic embryos and their subsequent conversion into plantlets[5]. Among the different explants tried till date, plumular tissues have proven to be the most responsive *in vitro* for both formation of embryogenic calli and induction of somatic embryos[3,4]. However, the efficiency of formation of somatic embryos has remained low even in plumular tissues[4].

All aspects of shaping and establishment of plant growth and development are governed by systemic signaling molecules and phytohormones. Plant receptor-like kinases (RLKs), residing in the plant plasma membrane, belong to the monophyletic interleukin-1 receptor-associated kinase (IRAK) or RLK/Pelle family[22]. They are composed of an extracellular ligand-binding domain, which are linked via transmembrane domains to cytoplasmic serine/threonine protein kinase domains[23]. On imposing conformational changes on RLKs, which may occur either through ligand binding to the extracellular domain or via stimulus-induced complex formation of RLKs with other proteins, an intracellular signal transduction cascade is initiated[24].

Among the different genes involved in regulation of somatic embryogenesis that have been identified till date, SERK has been well-studied and is demonstrated to play a key role in somatic embryogenesis in many plant species[5,6]. In addition to promoting transition of single somatic cells to an embryogenic stage, SERK has been known to be a marker of embryogenic competence[5]. Over-expression of *Arabidopsis* ortholog SERK1 resulted in enhanced formation of embryogenic cells in response to the growth regulator 2, 4-D[6], suggesting that SERK1-mediated signaling is involved in embryogenic cell formation. Although a basal level of expression of *SERK* is found in various rice organs/tissues, an increased higher expression pattern is detected in rice callus on induction of somatic embryogenesis[9]. On suppression of expression of *OsSERK1* in transgenic calli by RNA interference, a significant reduction of shoot regeneration rate (from 72% to 14% in the japonica rice Zhonghua11) has been reported. Overexpression of OsSERK1 results in an enhancement of the shoot regeneration rate in rice from 72% to 86%[9]. The *Arabidopsis* SERK3 gene has been identified as the Brassinosteroid Insensitive1 (BRI1)-Associated Kinase1 (BAK1)[25]. BRI1 has been shown to form heterodimers with SERK3/BAK1 in living cells[26], the interaction being dependent on the presence of brassinosteroids[27]. CLAVATA1 (CLV1) is an example of yet another receptor that has a role in maintaining the proper balance between undifferentiated cells and cells destined to differentiate into organs in the shoot apical meristem[28].

A detailed study of SERK in coconut might provide invaluable information on its activity, as well as clues to enhance the rate of somatic embryogenesis by induction of this gene in coconut, which has so far remained recalcitrant to *in vitro* culture. As an initial step, we carried out homology based modeling of a SERK protein from coconut, CnSERK. Plant growth regulators, especially auxins and cytokinins, are mainly involved in regulation of cell differentiation. Therefore, we carried out docking studies using 15 plant growth regulators to study their interaction dynamics with CnSERK. Candidate growth regulators displaying highest docking score with CnSERK were

then supplemented in the tissue culture media to study their ability *in vitro* to enhance the rate of embryogenic calli formation and induce somatic embryogenesis from coconut plumular explants

In majority of the LRR receptor kinases, the LRR domain is followed immediately by the transmembrane domain. However, in CnSERK, similar to most of the plant SERKs, these two domains are separated by a Pro-rich region containing a repeated SPP motif. The function of the SPP domain has been proposed to be two-fold: as a region to impart flexibility to the extracellular part of the receptor or in interaction with the cell wall[5,6]. SERKs possess only five LRRs in contrast to two of their main ligand binding receptors BRI1 and FLS2, which have 24 and 28 LRRs respectively[9,24,25]. Additionally, in BRI1, there is 70-amino acid island domain located between LLR21 and LRR 22, which is the minimal binding domain for brassinosteroids[29].

Receptor kinases have been classified into two families on the basis of amino acid residues (Ser and/or Thr or Tyr) that are phosphorylated. However, the kinase domain of proteins like SERK1 can transphosphorylate on Ser, Thr and Tyr residues and hence represent kinases with dual specificity[30]. In spite of the high conservation of kinase domain sequence of various plant SERKs, variations have been noticed both in the phosphorylation peptides and their modes of phosphorylation.

From the predictions of PONDR®VL-XT, CnSERK was found to be largely structured, with only two main segments of disorder in the region 206 to 246 and C-terminal residues from 556 to 629. This region of disorder has been identified earlier as a region which is essential for kinase phosphorylation activity *in vitro* and also functions of SERK1 in planta[31]. Phosphorylation sites, due to easy access of these sites, probably are usually seen in disordered regions of proteins[32]. Structuring in these disordered regions[33] and ability of these regions to undergo protein-protein interactions is influenced by phosphorylation[34]. There is wide variation in the number of phosphorylation sites between SERK1 and its closest paralogs *viz*., SERK2 and BAK1 (SERK3)[35]. In CnSERK, studies revealed the potential phosphorylation sites in the kinase domains as Ser306, Ser397, Ser418, Ser481, Ser485, Ser486, Ser565, Thr340, Thr349, Thr371, Thr373, Thr404, Thr462, Tyr479 and Tyr546.

Since the structure of SERK protein has yet to be elucidated, homology modeling is only method to elucidate the structure of given protein. Homology modeling uses previously solved structure as templates and requires only 30% sequence identity with sequence, whose structure has been deduced[36,37]. Since no single template could be found, a total of three prospective templates (PDB id: 3TL8_A, 4MN8_B and 4Q3G_A) were selected for modeling of based on their sequence identities with CnSERK[37]. Result of docking studies revealed that the amino acids were mainly involved in binding of ligands. Based on the docking scores, three growth regulators *viz*., adenine sulphate, 22(S), 23(S)-homobrassinolide and glutathione were selected for further experiments.

In vitro studies using plumular explants, designed and implemented based on the results of *in silico* studies revealed that glutathione was found to be better for somatic embryo formation, compared to other treatments. A thioldisulfide compound, glutathione is considered to be one of the most significant growth regulators, owing to its antioxidant action and possible effects on protein structure and function[38]. One of the prejudices of using activated charcoal in the medium is that it can adsorb essential plant growth regulators and some minerals[39]. This creates undefined culture condition which can lead to variable tissue response and non-reproducible result. The presence of 2, 4-D in the culture medium also has the tendency to induce browning in the cultures[38]. There is considerable evidence that reduced glutathione plays a vital role as an antioxidant in the defense systems of plants against environmental stress and known to relieve stress conditions, such as oxidation[40]. Browning of *Taxus baccata* cultures is reduced by supplementing B5 medium with 0.1 mM glutathione[41]. In apple culture, medium containing reduced glutathione (GSH) promotes callus growth[42], while in *Pistacia vera* shoot tip culture[43], GSH reduces the total phenolic compounds and significantly promotes growth. An addition of 0.1 mM GSH results in an increase in somatic embryo production in white spruce tissue culture, suggesting that GSH can induce growth of somatic embryo through distinct metabolic changes of pyrimidine nucleotides[44,45]. The role of glutathione in the maturation process of somatic embryo is also reported in *Pinus roxburghii*[46]. Recently, it has been reported that glutathione status is important to allow intracellular oxidation to activate pathogenesis-related phytohormone signaling pathways[47].

Brassinosteroid (BR) is considered as a plant growth regulator which is vital for normal plant

growth and development[48]. BRs are known to act synergistically with auxins and might be involved in the control of plant embryogenesis[49]. The study on the effect of BR in coconut plumular tissue culture has shown that pre-treatment of coconut explants with 22(S), 23(S)-homobrassinolide for three days significantly improves somatic embryogenesis[50]. The 24-epibrassinolide promotes cell division in the presence of 2,4-D and kinetin in Chinese cabbage protoplasts[51]. BRs are involved in various developmental processes and responses to biotic and abiotic stresses[52]. SERKs play a critical role in the early events of BR signaling likely via a reciprocal and sequential phosphorylation model[13]. Auxins stimulate BR perception by regulating the level of BR receptor[53].

Adenine sulphate incorporation into the culture medium has a beneficial effect on the explants of *Jatropha*, in association with other phytohormones[54]. In date palm, high rate of callogenesis is obtained in the presence of 2, 4-D and 40 mg/L adenine sulphate[55]. Adenine sulphate may act as a precursor of natural cytokinin[56]. Higher concentration of adenine sulphate (200-300 mg/L) has shown frequent basal callusing of the explants of *Picrorhiza scrophulariiflora*[57].

Conclusion

Coconut tissue culture has proved to be challenging, in spite of decades on research in this area. The 3-D structure of CnSERK might provide a better insight in understanding the structure and function of SERK in coconut tissue culture. The results of the present study provide invaluable leads on utilization of novel plant growth regulators, based on *in silico* and *in vitro* studies, which possess potential to be used for improving *in vitro* induction of embryogenic calli and somatic embryogenesis in coconut.

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