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Tissue Specific Localization Expression of *SOC1* Gene in Oil Palm

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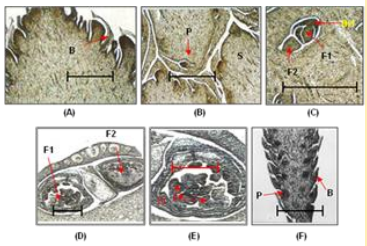
Graphical abstract



Abstract

The oil palm industry has been affected by abnormality in its clonal palm. Oil palm abnormality which arose from *in vitro* regeneration was first detected during flowering process. In this study, localized expression of an oil palm homologue of *SOC1* gene was investigated using *in situ* RNA hybridization. Tissue specific localization expression of *OPSOC1*and *OPSOC1-3'* showed that *SOC1* is expressed in both normal and abnormal flower. The gene is highly expressed in abnormal oil palm flower throughout flower initiation and development. The role of *SOC1* in inducing floral organ and its expression pattern provides a better understanding of regulation of *OPSOC1* in normal and abnormal oil palm flower.

Keywords: *SOC1*; oil palm; flowering; *in situ* RNA hybridization

Abstrak

Industri kelapa sawit telah terjejas disebabkan oleh ketaknormalan klon kelapa sawit. Ketaknormalan kelapa sawit yang berpunca daripada penjanaan semula *in vitro* pertama kali dikesan semasa proses pembungaan. Di dalam kajian ini, ekspresi setempat homolog gen *SOC1* daripada kelapa sawit disiasat menggunakan penghibridan *in situ* RNA. Ekspresi setempat dalam tisu khusus pada *OPSOC1* dan *OPSOC1-3'* menunjukkan bahawa *SOC1* diekspresikan dalam kedua-dua bunga normal dan tidak normal. Gen ini diekspresikan dengan tinggi dalam bunga tidak normal sepanjang pembentukan dan perkembangan bunga. Fungsi *SOC1* dalam mendorong pembentukan organ bunga dan corak ekspresinya menyediakan pemahaman regulasi *OPSOC1* di dalam bunga kelapa sawit normal dan tidak normal yang lebih baik.

Kata kunci: *SOC1*; kelapa sawit; pembungaan; penghibridan *in situ* RNA

1.0 INTRODUCTION

In most crops, the control of flowering is an important aspect of growth and development. Flowering is often the first introductory step to fruit formation. In addition to being a fundamental part of the plants reproduction system, both flowers and fruit are also an integral part of seed production. Study on physiological background and flowering of oil palm enable the determination of factors influencing flowering process. Nonetheless, floral morphogenesis abnormality induced by *in vitro* regeneration [1,15] has hampered oil palm micropropagation. Classification of normal and abnormal oil palm flower development was proposed in 9 stages. The stages of floral development facilitate study on tissue specific expression of flowering genes. Cells and tissues in the plant organ systems are anatomically similar. To study floral-specific genes, isolation of mRNA from specific tissue without getting the samples contaminated with mRNA from the surrounding tissues is often a challenge.[13] The ability to detect nucleic acid *in situ* will enable determination of spatial and temporal expression patterns of flowering genes. It can be used to identify sites of gene expression, whereby the tissue distribution of expression, cellular specificity as well as temporal expression pattern of the flower-specific genes can be determined. This analysis can enable putative function of genes associated with specific cell types to be postulated. Additionally, the sensitivity of *in situ* RNA hybridization allows determination of gene expression at a specific location of a cell or tissue. Examination of *OPSOC1*, a

flowering gene from oil palm provides an insight during normal and abnormal flower formation of the plant. *SOC1* (*SUPPRESSION OF OVEREXPRESSION OF CONSTANS 1*) is a meristem and organ identity gene which acts downstream during transition to flowering process by inducing the formation of floral organs.[9-10] This study was conducted to provide an understanding of regulation of SOC1 in abnormal oil palm flower and its influence on floral abnormality.

2.0 EXPERIMENTAL

2.1 Plant Materials

The oil palm flowers (spikelets & inflorescences) were obtained from MPOB's plantation from Perak. They were divided according to their 9 developmental stages and later cut into 1 cm^2 pieces.

2.2 Probe Preparation

OPSOC1 floral gene from oil palm clones obtained from MPOB were transformed in DUAL TOPO kit (Invitrogen) as per manufacturer's instruction. A standard labeling reaction was then carried out as instructed using the DIG RNA Labeling Kit (Boehringer Mannheim). The probe was then resuspended in 50% formamide and kept at –20ºC prior to use.

2.3 Sample Preparation

The flower samples were fixed freshly into EAF fixative (50% ethanol, 5% acetic acid, 10% formamide) followed by dehydration in 70%, 96% and 100% ethanol for 30 min each at room temperature. The dehydration step was continued with incubation in 25% ethanol; 75% xylene, 50% ethanol; 50% xylene, 75% ethanol; 25% xylene for 30 min each and 3X 15 min in 100% xylene at room temperature. The samples were then infiltrated in 20 ml 100% xylene with 20 pieces of paraffin chips and incubated overnight at 42ºC. After infiltration, the samples were embedded in steel mold according to desired orientation, installed the holder and kept at 4ºC until further use. To fix the sample on the poly-L-lysine slides, the sample was cut between 7–12 µm using microtome. The slides were warmed at 45ºC on a slide-drying bench and before the paraffin ribbons (shiny side down) were placed on the slide, a few drops of DEPC-dH_2O were put onto the slide. Excess water was discarded by sipping it with clean tissue, the slides were left to dry for 1 minute followed by further drying in 42ºC oven overnight and then kept at 4ºC.

2.4 *In Situ* RNA Hybridization: Prehybridization and Hybridization

The samples slides were dehydrated 2X 10 min histoclear or xylene, 2X 1min 100% Ethanol (EtOH), 1 min 95% EtOH, 1 min 90% EtOH, 1 min 80% EtOH, 1 min 60% EtOH, 1 min 30% EtOH and 1 min dH_2O; washed 15 min in 2X SSC (RT); incubated 30 min at 37ºC in proteinase solution (1µg/ml proteinase K in 100 mM Tris-HCl pH 8.0, 50 mM EDTA); rinsed 2 min in 2 mg/ml Glycine in PBS (RT), 2X 2 min PBS (RT); incubated 10 min in 4% Paraformaldehyde pH 7.0 (RT); rinsed 2X 5 min in PBS; incubated 10 min in 0.1M Triethanolamine pH8.0 + Acetic anhydride; rinsed 2X 5 min in PBS; dehydrate 30 sec 30% EtOH, 30 sec 60% EtOH, 30 sec 80% EtOH, 30 sec 90% EtOH, 30 sec 95% EtOH, 2X 30 sec 100% EtOH and stored (prehybridized) in container containing EtOH for several hours at 4ºC.

For hybridization, a sufficient amount of probe was added to 50% formamide to make up into 40 µl (each pair of slides) and heated up at 80ºC for 2 min. The probe was transferred to ice immediately and 160 µl of hybridization solution (for 5 pairs of slides: 100 µl 10X *in situ* salts, 400 µl deionized formamide, 200µl 50% dextran sulfate, 20µl 50X Denhardts solution, 10µl tRNA [100 mg/ml] and 70 µl DEPC dH_2O) was added to the probe and the mixture was gently mixed to avoid bubbles. The probe was applied to the slides by spreading approximately 150µl probe/ hybridization solution to one slide and then sandwiching the other slide onto it. The slides were put above wetted paper towels (wet by dH_2O) in plastic container using glass pipettes and hybridized overnight at 55ºC.

2.5 *In Situ* RNA Hybridization: Post-Hybridization Steps

The slides were treated as follows: incubated in 0.2X SSC for 1 hour (55ºC) and repeated with a new solution; washed in NTE (0.5M NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA) 2X 5 min at 37ºC; incubated in RNase solution (20 µg/mL RNase A in NTE) 30 min (37ºC); washed 2X 5 min in NTE (37ºC); incubated 1 hour in 0.2 X SSC (55ºC); washed in PBS for 5 min at RT (or overnight in 4ºC); washed 45 min in Blocking solution A (1% in TBS [100 mMTris-Hcl pH 7.5, 150 mM NaCl]) at RT and another wash in Blocking solution B (1% BSA, 0.3% Triton X-100 in TBS) for 45 min at RT.

2.6 *In Situ* RNA Hybridization: Immunological Detection

The detection step involved incubating the slides (sandwiched) in diluted anti-DIG Fab Fragment antibody where the antibody was diluted 1:1250 in the Blocking solution B for 2 hours at RT or overnight at 4ºC. The slides were washed again in Blocking solution B to remove unbound antibody for 4X 15 min at RT. The final wash was done in Buffer C (100 mM Tris-HCl pH 9.5, 50mM $MgCl_2$, 100 mM NaCl) for 15 min at RT to ensure all detergent was washed off. To develop the hybridization signal, the slides were stained with NBT/BCIP staining solution in 10 ml Buffer C). The solution was applied to the slide and it was sandwiched with another slide, where it was kept at RT in moistened plastic container (as in hybridizing step) and dark environment for 3 to 4 days, depending on the color formation. To stop the reaction, the slides were dehydrated as follows: 5 sec 30% EtOH, 5 sec 50% EtOH, 5 sec 70% EtOH, 5 sec 85% EtOH, 5 sec 95% EtOH, 2X 5 sec 100% EtOH and 2X 5 sec histoclear/ xylene. Slides were air dried and kept in a slide box prior to viewing under light microscopy.

3.0 RESULTS AND DISCUSSION

3.1 Expression Pattern of *OPSOC1* was Observed throughout Flower Development

OPSOC1 (AF 207699) is highly homologous to *AGL20* of *Arabidopsis* (AY 007726). It is one of the well known floral pathway integrator genes.[12,14] Generally, *SOC1* is induced in the meristem by the *FLOWERING LOCUS T (FT*) to activate flowering.[16,17] Among the roles played by *SOC1* in *Arabidopsis* are the regulation of flowering time, floral patterning and floral meristem determinacy.[8,10] As a member of MADS-box family encoding a conserved MADS box protein among angiosperms [2,3,5,6,11] similar *SOC1* characteristics are also reported in other

plant species. It was postulated that *SOC1* is likely to play a role as a general regulator in organogenesis [7] during plant development.

The full length (*OPSOC1*) and 3' end of *OPSOC1 (OPSOC1-3')* were used as probes for *in situ* hybridization (ISH). The difference between these 2 probes is that the full length contains the MADS box whereas the 3'end probe does not and acts as a gene specific probe. The MADS box genes are composed of 4 regions; M, I, K and C and are likely to play an important role in floral diversification because they regulate the major steps in floral morphogenesis. The MADS box is a conserved region at the 5' end of all plant MADS box genes encoding a DNA-binding domain.[4] The *OPSOC1-3'* may function as a transcriptional activation domain as it comprise of only the C-region at the carboxyl-terminal of the MADS-domain proteins.

In sections probed with the full length *OPSOC1*, the signal was detected mainly in abnormal flowers with the first expression observed in stage 2 of the abnormal flower. Summary of stages of oil palm flowering is as in Table 1. Expression was observed on the meristem (Figure 1(A)) but from then onwards, no expression was observed until stage 4 in the abnormal male flower (Figure 1 (F)). Strong expression was observed in the male flower primordia as well as in the bracts surrounding them (Figure 1 (B)). Expression continued in stage 5 from both abnormal male and female flowers where the signal was also strong on the flower primordia and bracts (Figure 1 (C)). In the larger abnormal female flower of stage 8, strong expression was detected on the stamens within the internal carpels of staminate flowers (Figure 1 (D)) and the supernumerary carpels of stage 9 abnormal female flower (Figure 1 (E)). The expression of this gene was not observed in any stages of both normal male and female flower.

Table 1 Summary of stages of oil palm flower development

Normal flowers	*Abnormal flowers*
Stage 1: Inflorescence meristem develops, giving primary axis. Length of inflorescence < 1 mm.	**Stage 1**: Inflorescence meristem develops, giving primary axis. Length of inflorescence < 0.5 mm.
Stage 2: Primary axis differentiates to form spikelet primordia. Inflorescence length is about 1 mm – 5 mm.	**Stage 2**: Primary axis differentiates to form spikelet primordia. Inflorescence length is < 1 mm.
Stage 3: Bracts developing around the spikelet. This is the sex definition stage where the female & male spikelets carry bracts of a different number & shape. Length is about 1 cm – 1.5 cm	**Stage 3**: Bracts developing around the spikelet. Length is about 0.5 mm – 1 cm.
Stage 4: Emergence of flower primordia. Inflorescence length is about 2.0	**Stage 4**: Emergence of flower primordia. Inflorescence length is about 1.5
Stage 5: Emergence of male flowers in a triad of female inflorescences. Male flowers in the male spikelet will also develop at this stage. Inflorescence length is about 3.1 – 4.9 cm.	**Stage 5**: Emergence of male flowers in a triad of female inflorescence. Inflorescence length is about 2.1 – 4.0 cm.
Stage 6: Formation of the female flower in the female inflorescence triad. Male flower of the triad develop young stamens. Inflorescence length is about 5.0	**Stage 6**: Formation of female flowers in the female inflorescence triad. Inflorescence length is about 4.1 – 4.9 cm.
Stage 7: Development of young carpels on a female flower. Stamen primordia can be detected but do not develop further. Anther develops on male flower of the triad. Inflorescence length is about 7.0 - 8.9 cm.	**Stage 7**: Development of supernumerary carpels (SC). Inflorescence length is about 5.0 – 6.9 cm.
Stage 8: Carpels are fully developed & ovule primordial originate. Anthers are fully developed and pollen are being produced. Inflorescence length is about 9.0 – 14.9 cm.	**Stage 8**: Internal carpels develop fully while stamens of abnormal male flower developed into carpel-like structure. Inflorescence length is about 7.0 – 12.5 cm.
Stage 9: Development of ovule in female flower. In male flower, the pollen fully developed and are dividing. Inflorescence length is 15.0 cm and above.	**Stage 9**: Supernumerary carpel developed fully and stamens of male flower develop into carpel-like structure. Inflorescence length is 13.0 cm and above.

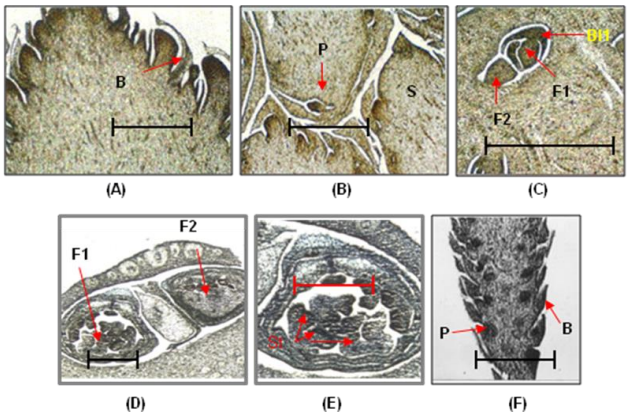


Figure 1 Expression of OPSOC1 was obvious in abnormal female flowers as compared to abnormal male flower. (A) Stage 3,female; (B) stage 4, female; (C) stage 5, female; (D) carpels of stage 8, female; (E) supernumerary carpel of stage 9 and (F) stage 4, male. Bar indicates 3mm in length. B=Bract, F=Staminate flower, M=Meristem, P=Primordia, S=Spikelet, St=Stamen

Similar expression was observed at the first stage of flowering in both normal and abnormal flowers with *OPSOC1-3'* (Figure 2). In abnormal palms, the expression extended in young leaves and meristems with consistent expression on flower primordia at stage 2, 3, 4 and 7 (Figure 2 (A until D)). Expression in normal flowers however, could not be detected after the first stage of flowering (Figure 2 (E and F)).

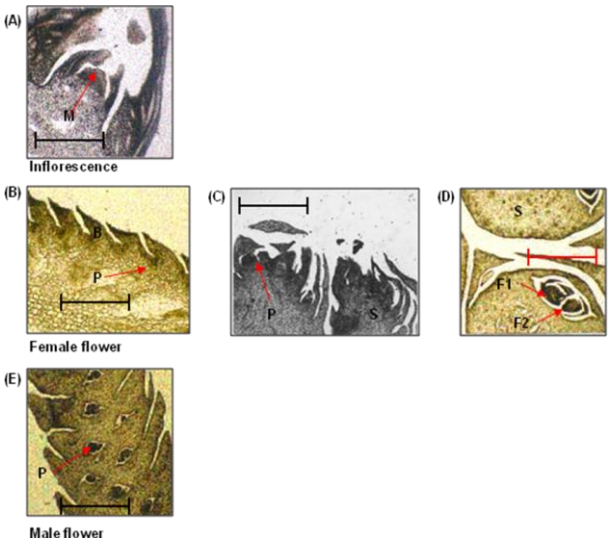


Figure 2 Expression of OPSOC1-3' in normal and abnormal flower. (A) Abnormal inflorescence; (B) normal female, stage 3; (C) abnormal female, stage 3 (D) normal female, stage 5 and (E) abnormal male, stage 5. Bar indicates 3mm in length. B=Bract, Bl=Bracteole, F=Staminate flower, M=Meristem, P=Primordia, S=Spikelet

This expression pattern proved the requirement of *SOC1* throughout flower development of both normal and abnormal palms. Postulation was made that both normal and abnormal palms share similar floral induction and maintenance. Additionally, studies on SOC1 protein suggested its role in general regulation of floral patterning and determinacy in plant development.[9-10] *SOC1* gene is known to perform its role in the apical meristems during flowering however; the gene is also expressed in leaves during vegetative phase independently of the flowering process. Its function to induce flowering have been supported by various studies on *soc1* mutants whereby in the absence of the gene plants display late-flowering characteristics.[5,10] It is of interest if a study on SOC1 protein could be applied to both normal and abnormal palm. Similar SOC1 characteristics were documented in other plant species [2,3,5,6,11] where its role as a general regulator in organogenesis during plant development was suggested.[7] Information obtained from protein expression study of SOC1 in oil palm could be compared to that of its gene and could further enhance the regulation of SOC1 at transcript and protein level.

■4.0 CONCLUSION

An expression study of *SOC1* in oil palm via *in situ* RNA hybridization showed that the gene is required throughout flower development of both normal and abnormal palms. Further studies on SOC1 protein and its expression in both normal and abnormal palm would enable comparison of the SOC1 protein expression and function to those obtained from *SOC1* gene. Information provided by studies on regulation of flowering at the transcript and protein level combined with *in vitro* micropropagation could be applied to minimized clonal abnormality in oil palm.

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