DOI: 10.1007/s10535-013-0325-1 BIOLOGIA PLANTARUM 57 (3): 597-600, 2013

BRIEF COMMUNICATION

Characterization of RAM to SAM transitions in *Selaginella microphylla* grown *in vitro*

S. BANDYOPADHYAY[2], K. NANDAGOPAL[3], and T.B. JHA[1]*

Department of Botany[1] and Department of Biotechnology[2], Presidency University, 86/1 College Street, Kolkata-73, West Bengal, India
Department of Genetics, University of Calcutta, 35 B.C. Road, Kolkata-19 West Bengal, India[3]

Abstract

In vitro morphogenetic ability of plant cells has been demonstrated in diverse species of angiosperms and gymnosperms but no such report is available in the genus *Selaginella* till date. We have established an *in vitro* morphogenic root culture where indole butyric acid (IBA) induced profuse branched and unbranched roots in *Selaginella microphylla*. We observed inter-convertibility of root apical meristem (RAM) to shoot apical meristem (SAM) in presence of IBA and show that intact roots are also capable of transformation. Friable callus was obtained from roots on prolonged (~50 weeks) root cultures. By isolating total RNA from each of the developmental stages, we performed cDNA synthesis followed by random amplification, sequencing, and *BLAST* analysis of differentially expressed transcripts to correlate morphological events with the changes on molecular level. The results reveal sequence matches to genes involved in diverse cellular processes, such as transcription, translation, photosynthesis, replication, secondary metabolism, stress response, and plant defense suggesting ancient origins of such proteins and the evolutionary conservation of biological function.

Additional key words: *BLAST* analysis, indole butyric acid, RAPD, root culture, sequencing.

The shoot apical meristem (SAM) and root apical meristem (RAM) are intimately involved in control of plant growth and development. Both RAM and SAM are comprised of stem cell populations, some of which undergo differentiation; a small group preserves their undifferentiated identity during the whole life span. Thus, the continuum of differentiation, de-differentiation, and re-differentiation manifests in different parts of a plant in a restricted manner. *In vitro* plant cell culture systems have, however, proved conclusively that almost any cell can behave like a stem cell, undergo de-differentiation and re-differentiation through optimization of culture conditions and even lead to the regeneration of whole plants (George *et al*. 2008). This is proof of enormous developmental plasticity that exists in plant cells (Lijsebettens and Montagu 2005). Recent advances in plant genetics and molecular biology have yielded new insights into the roles of genes controlling cell behavior in the two primary meristems and have revealed similarities between the molecules and mechanisms hinting at their shared evolutionary origin (Stahl and Simon 2010).

The genus *Selaginella* (spike moss) is an enigma in the plant kingdom. It has survived virtually unchanged for hundreds of millions of years (Banks 2009). Sequencing the genome of *Selaginella moellendorffi,* the smallest genome (~110 Mbp) within the plant kingdom reported so far, has provided a huge thrust in *Selaginella* research. New genomic information has the potential to provide critical information about the evolution of land plant genes and genomes. Although there has been progress in identification of key genes that regulate *in vitro* organogenesis and embryogenesis, the studies have focused on only few species (Bao *et al*. 2009) and molecular data are mostly unavailable.

When grown in Knop's medium without any growth regulator, *Selaginella willdenovii* undergoes a unique

Received 27 June 2012, *accepted* 28 January 2013.
Abbreviations: IBA - indole butyric acid; NCBI - National Centre for Biotechnology Information; PCR - polymerase chain reaction; RAM - root apical meristem; RAPD - randomly amplified polymorphic DNA; SAM - shoot apical meristem.
Acknowledgements: The work was supported by the Department of Biotechnology Research Associateship programme (DBT-RA), Govt. of India.
* Author for correspondence; fax: (+91) 033 2241 2738, e-mail: tbjha2000@yahoo.co.in

transition of RAM to SAM (Wochok and Sussex 1976). However, no shoot was obtained with plant growth regulators. Similarly in *Selaginella microphylla*, an Indian species with a geographical distribution that is distinct from *S. willdenovii*, we have reported *in vitro* morphogenic aspects of plant regeneration and a role of IBA in initiating root culture and subsequently re-differentiation of SAM from RAM (Jha *et al*. 2012). In the present report, *in vitro* morphological and molecular evidences of transition of RAM to SAM in different developmental stages are presented.

In vitro grown roots on rhizophores were used to initiate root cultures. Roots were placed in a half strength Murashige and Skoog (MS) medium with 3 % (m/v) sucrose and varying concentrations of indole butyric acid (IBA; 2.0 - 9.8 µM). Other culture conditions were the same as reported earlier (Jha *et al*. 2012). The optimum IBA concentration was 9.8 µM. The growth of roots was monitored from 3 weeks onwards up to 30 weeks through sub-culturing at 5 - 6 week intervals.

Total RNA was isolated from *in vitro* cultures representative of four different developmental stages of *S. microphylla*, namely differentiated root cultures, root cultures with green swollen RAM and re-differentiated SAM, developing green shoots, and undifferentiated mass of cells (callus), with the help of RNeasy plant mini kit (*Qiagen*, Hilden, Germany). A DNase digestion was also performed to obtain highly pure RNA samples. Double stranded complementary DNAs (cDNAs) were synthesized with *Omniscript* reverse transcript polymerase chain reaction (RT-PCR) kit and oligo-dT primers (*Fermentas*, Burlington, Canada). For RAPD analysis, fifteen random decamer primers, obtained from *Operon Technologies* (Alameda, USA**;** OPA 1 - 15), were used in reactions containing 200 µg cDNA, 10× PCR buffer, 2 mM deoxyribonucleotide triphosphates (dNTPs), 2 µM random primer (5′-GAAACGGGTG-3′ and 5′-AGTCAG CCAC-3′) and 3 U of *Taq* DNA polymerase (*Fermentas*) in a total volume of 0.02 cm^3. The PCR conditions were as follows: 94 °C for 5 min, then 35 cycles of 94 °C for 30 s, 38 °C for 30 s, 72 °C for 1 min, and finally an extension at 72 °C for 7 min. The amplified DNA products were separated on 2 % (m/v) agarose gel. Several bands from three developmental stages were randomly gel eluted and re-amplified under the same PCR condition as above and sequenced directly. Sequences were matched with *NCBI* database with the help of *BLASTn,x* and plant genome database separately (to enhance coverage) keeping default parameters.

Roots in *Selaginella* are never produced directly on stems, they are produced on rhizophore and root apical meristems (RAM) are always bifurcated. However, within 5 - 8 weeks of *in vitro* culture on half strength MS medium with IBA (9.8 µM), we obtained direct induction of secondary roots on primary root tissues which slowly transformed into a yellowish white root masses (Fig. 1*A*,*B*). It is noted that *in vitro* grown roots were not dependant on rhizophore and manifested with or without bifurcation of RAM. Our result is an indication that trait(s) existed in RAM of *S. microphylla* for development of non-bifurcated normal roots, an important feature of higher vascular plant. Well-grown root cultures were maintained in the same hormonal concentration and 5 - 10 % of root apices after 8 to 10 weeks

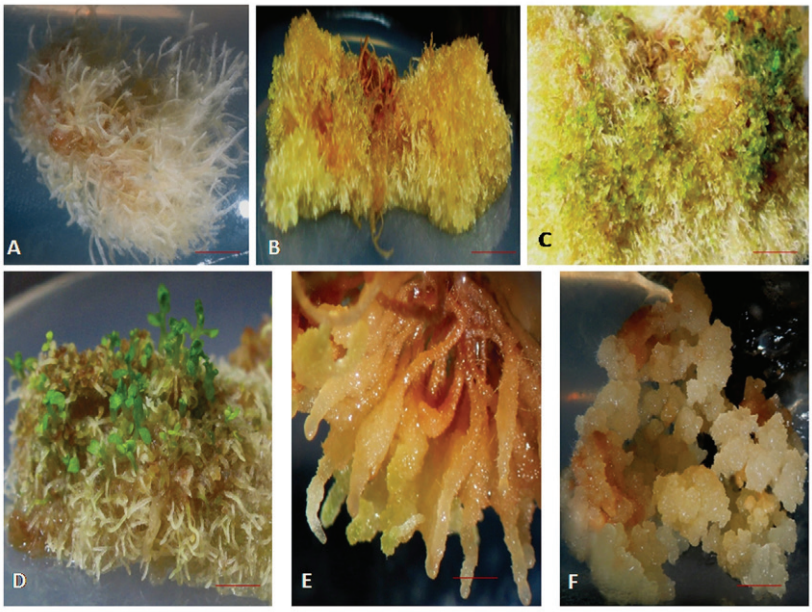

Fig. 1. *A* - 10-week-old root culture of *S. microphylla*, *B* - 15-week-old root mass, *C* - 20-week-old root culture showing numerous oval and green root apical meristems and re-differentiation of shoot apical meristems, *D* - differentiation of shoots in 30-week-old culture, *E* - induction of callus in root culture older than 50 weeks, *F* - development of white friable callus in root culture older than 50 weeks. *Bar* = 0.5 cm for all figures.

of culture in IBA (9.8 μM) showed swelling (oval shaped) of RAM, and non-green RAM portions gradually converted to green SAM like structure (Jha *et al* 2012, Fig. 1*C*). We consider this was the stage of transition of RAM to SAM, and confirmation was established when we documented the differentiation of green shoots with micro leaves from the green oval zones (Fig. 1*D*). Our results reconfirm the ability of inter convertibility of RAM to SAM as reported by Wochok and Sussex (1976) in another species of *Selaginella*. In our studies, no other growth regulator besides IBA was used and more studies are required to establish the role of IBA as signal molecule for redetermination of RAM to SAM in intact roots as well as callus formation in aged cultures. More than 50-week-old cultures developed friable callus in the same medium (Fig. 1*E*,*F*). Callus production has not been reported before in any species of *Selaginella.* Therefore, callus masses were also used for molecular analysis.

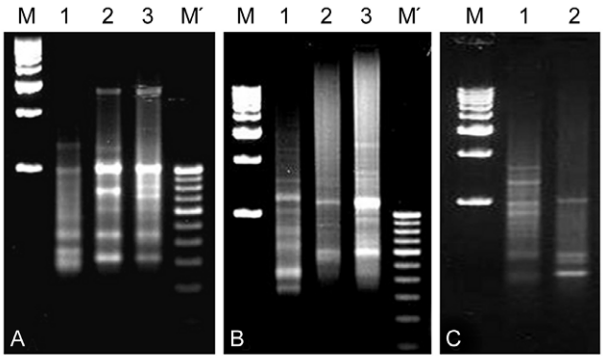

Fig. 2. The c-DNA RAPD profile from different developmental stages of *in vitro* grown *S. microphylla* using two primers (5´-GAAACGGGTG-3´ and 5´-AGTCAGCCAC-3´). *A*, *B*: *lanes* 1 - root culture (see Fig. 1*A*), *lanes* 2 - root cultures with green swollen RAM and re-differentiated SAM (see Fig. 1*C*), *lanes* 3 - tiny green shoots (see Fig. 1*D*), *lanes* M and M' correspond to lambda DNA *HindIII* digest and 100 bp DNA ladder, respectively. *C*: *lanes* 1 and 2 - undifferentiated mass of cells (see Fig. 1*F*), *lane* M - corresponds to lambda DNA *HindIII* digest.

The cDNA-RAPD technique in conjunction with DNA sequencing permits rapid identification of functional genes in a cost effective manner (Chen *et al*. 2005, Nimbhalkar *et al*. 2006, Agarwal *et al*. 2008). The cDNA-RAPD profile generated in our studies with 15 random primers produced several amplicons; 23 bands from four developmental stages were randomly gel eluted and re-amplified under the same PCR conditions followed by direct sequencing. This demonstrates that the identification of functionally important genes that governed developmental decisions was possible in S. *microphylla* (Fig. 2).

The identification of RNA polymerase β-subunit expect value (E) = 8×10^{-35} among transcripts expressed in differentiated green oval-shaped apices simulating SAM, and also in shoots indicates a pivotal role for transcription control in the specific developmental stages and not detected in non green roots and callus. Furthermore, a sequence match with elongation factor TF-IIS (E = 3.9) reveals a role for transcript elongation factors that facilitated RNA polymerase-mediated transcription bypassing blocks to mRNA synthesis (Mortensen *et al*. 2011). A decrease in TF-IIS expression is known to reduce seed dormancy in *Arabidopsis* (Grasser *et al*. 2009), an intriguing observation in light of the fact that *in vitro* cultures of *Selaginella* are very slow growing. The evolutionary significance of the WRKYGQK peptide sequence containing WRKY family (E = 0.5) of transcription factors which co-ordinate activation and repression of multiple developmental processes (Rushton *et al*. 2010) is suggested, as their potential role is combating abiotic stress (Chen *et al*. 2011) encountered *in vitro*. A sequence match with cysteine-histidine rich domain containing protein (E = 1.8) further suggests that it might be similar to zinc-finger containing transcriptional co-activators such as CBP (Newton *et al*. 2000, Kalkhoven *et al*. 2002). The *Selaginella* root culture model described in this study could thus prove useful in discerning evolution of the transcription machinery in land plants.

Retrotransposon-like sequences (hypothetical protein, E = 0.73; FA553, E = 0.27) were identified in our screen. The insecticidal toxin protein (E = 0.89) and cold shock protein (E = 0.19) are representative of other sequences identified in the functional categories of plant defense and stress responses. A sequence match with metazoan target of rapamycin (TOR) serine-threonine kinase (E = 2.5) points to the existence of evolutionarily conserved signal transducing pathways in *Selaginella* that are related to growth and autophagy in plants (Liu and Bassham 2010, Dobrenel *et al*. 2011).

We also inferred sequence similarity to enzymes such as α-1,4-fucosyltransferase (E = 1.1), a component of biochemical pathways mediating shoot tissue differentiation. The ribosomal S8 protein gene clusters with other ribosomal genes in the chloroplast genomes of maize and tobacco (Tanaka *et al*. 1986, Markmann-Mulisch and Subramanian 1988). Obtaining a good match to the ribosomal S8 sequence (E = 6×10^{-37}) in white roots of *Selaginella* cultures was therefore unexpected and the finding indicates a significant role for translation control in the process of differentiation. A very significant sequence match to a chlorophyll-binding protein of photosystem II, CP47 (E = 3×10^{-106}), was revealed among *Selaginella* transcripts expressed in differentiated green oval shaped apex simulating shoot apical meristems. This suggests that the assembly of inner antenna light-harvesting proteins of oxygenic photosynthesis (Bricker *et al*. 2001, Zhang *et al*. 2007, Boehm *et al*. 2011) is also evolutionarily conserved and perhaps recapitulated *in vitro* by the *Selaginella* cultures.

Sequencing cDNA fragments from the callus stage of *S. microphylla* revealed totally different molecular identity. We obtained sequence similarity with Mob1-like proteins (E = 5×10^{-16}) and phenylalanine ammonia lyase (PAL, E = 2×10^{-40}), the former ones are known to be

involved in cell proliferation and are localized in the cell division plane during cytokinesis (Citterio *et al*. 2006), whereas PAL is reported to be active in early seedling development in vascular tissues of roots and leaves but not in the root tip or shoot apical meristems in *Arabidopsis* (Ohl *et al*. 1990).

There are several reports describing *in vitro* molecular developmental biology of the model plant *A. thaliana* (Che *et al*. 2006) but few reports, if any, on the group of lower plants like *S. microphylla*. Our *in vitro* studies have reported for the first time RAM to SAM transitions in *S. microphylla* using morphological and molecular analysis. Our results indicate and support operation of similar regulatory molecules and mechanisms in RAM and SAM as well as their shared evolutionary origins (Jiang and Fieldman 2005, Stahl *et al.* 2009). This knowledge could stimulate future studies related to evolutionary significance of gene regulatory mechanisms in higher plants.

References

Agarwal, M., Shrivastava, N., Padh, H.: Advances in molecular marker techniques and their application in plant sciences.- Plant Cell Rep. **27**: 617-631, 2008.

Banks, J.A.: *Selaginella* and 400 million years of separation. - Annu. Rev. Plant Biol. **60**: 223-238, 2009.

Bao**,** Y., Dharmawardhana**,** P., Mockler, T.C., Strauss, S.H.: Genome scale transcriptome analysis of shoot organo-genesis in *Populus*. - BMC Plant Biol. **9**: 132, 2009.

Boehm, M., Romero, E., Reiseinger, V., Komenda, J., Yu, J., Eichacker, L.A., Dekker, J.P., Nixon, P.J.: Investigating the early stages of photosystem II assembly in *Synechocystis* sp.PCC6803: isolation of CP47 and CP43 complexes. - J biol. Chem. **286**: 14812-14819, 2011.

Bricker, T.M., Lowrance, J., Sutton, H., Frankel, L.K.: Alterations of the oxygen-evolving apparatus in a (448)Arg-(448)S mutant in the CP47 protein of photosystem II under normal and low chloride conditions. - Biochemistry **40**: 11483-11489, 2001.

Che, P., Lall, S., Nettleton, D., Howell, S.H.: Gene expression programs during shoot, root, and callus development in *Arabidopsis* tissue culture. - Plant Physiol. **141**: 620-637, 2006.

Chen, L., Song, Y., Li, S., Zhang, L., Zou, C., Yu, D.: The role of WRKY transcription factors in plant abiotic stresses. - Biochim. biophys. Acta **1819**: 120-128, 2011.

Chen, Y.H., Tsai, Y. J., Huang, J.Z., Chen, F.C.: Transcription analysis of peloric mutants of *Phalaenopsis* orchids derived from tissue culture. - Cell Res. **15**: 639-657, 2005.

Citterio, S., Piatti, S., Albertini, E., Aina, R., Varotto, S., Barcaccia, G.: Alfalfa Mob1-like proteins are involved in cell proliferation and are localized in the cell division plane during cytokinesis. - Exp. cell. Res. **312**:1050-1064, 2006.

Dobrenel, T., Marchive, C., Sormani, R., Moreau, M., Mozzo, M., Montane, M.H., Menand, B., Robaglia, C., Meyer, C.: Regulation of plant growth and metabolism by TOR kinase. - Biochem. Soc. Trans. **39**: 477-481, 2011.

George, E.F., Hall, M.A., De Klerk, G.-J. (ed.): Plant Propagation by Tissue Culture. Volume 1. The Background. 3rd Ed. - Springer, Dordrecht 2008.

Grasser, M., Kane, C.M., Merkle, T., Melzer, M., Emmersen, J., Grasser, K.D.: Transcript elongation factor TFIIS is involved in *Arabidopsis* seed dormancy. - J. mol. Biol. **386**: 598-611, 2009.

Jha, T.B., Mukherjee, S., Basak, A., Adhikari, J.*: In vitro* morphogenesis in *Selaginella microphylla* (Kunth.) Spring. - Plant Biotechnol. Rep. DOI 10.1007/s11816-012-0255-y 2012.

Jiang, K., Feldman, L.J.: Regulation of root apical meristem development. - Annu. Rev. cell. dev. Biol. **21**: 485-509, 2005.

Kalkhoven, E., Teunissen, H., Houweling, A., Verrijzer, P.C., Zantema, A.: The PHD type zinc finger is an integral part of the CBP acetyltransferase domain. - Mol. cell. Biol. **22**: 1961-1970, 2002.

Lijsebettens, M.V., Montagu, M.V.: Historical perspectives on plant developmental biology. - Int. J. dev. Biol. **49**: 453-465, 2005.

Liu, Y., Bassham, D.C.: TOR is a negative regulator of autophagy in *A. thaliana.* - PLOS One **5**: e11883, 2010.

Markmann-Mulisch, U., Subramanian, A.R.: Nucleotide sequence and linkage map position of the genes for ribosomal proteins L14 and S8 in the maize chloroplast genome. - Eur. J. Biochem. **170**: 507-514, 1988.

Mortensen, S.A., Sønderkær, M., Lynggaard, C., Grasser, M., Nielsen, K.L., Grasser, K.D.: Reduced expression of the *DOG1* gene in *Arabidopsis* mutant seeds lacking the transcript elongation factor TFIIS. - FEBS Lett. **585**: 1929-33, 2011.

Newton, A.L., Sharpe, B.K., Kwan, A., Mackay, J.P., Crossley, M.: The transactivation domain within cysteine/histidine-rich region 1 of CBP comprises two novel zinc-binding modules. - J. biol. Chem. **275**: 15128-15134, 2000.

Nimbalkar, S.B., Harsulkar, A.M., Giri, A.P., Sainani, M.N., Franceschi, V., Gupta, V.S.: Differentially expressed gene transcripts in roots of resistant and susceptible chickpea plant (*Cicer arietinum* L.) upon *Fusarium oxysporum* infection. - Physiol. mol. Plant. Pathol. **68**: 176-188, 2006.

Ohl, S., Hedrick, S.A., Chory, J., Lamb, C.J.: Functional properties of a phenylalanine ammonia lyase promoter from *Arabidopsis.* - Plant Cell **2**: 837-848, 1990.

Rushton, P.J., Somssich, I.E., Ringler, P., Shen, Q.J.: WRKY transcription factors.- Trends Plant Sci. **15**: 247-258, 2010.

Stahl, Y., Simon, R.: Plant primary meristems: shared functions and regulatory mechanisms. - Curr. Opin. Plant Biol. **13**: 53-58, 2010.

Stahl, Y., Wink, R.H., Ingram, G.C., Simon, R.: A signaling module controlling the stem cell niche in *Arabidopsis* root meristems. - Curr. Biol. **19**: 909-914, 2009.

Tanaka, M., Wakasugi, T., Sugita, M., Shinozaki, K., Sugiura, M.: Genes for the eight ribosomal proteins are clustered on the chloroplast genome of tobacco (*Nicotiana tabacum*): similarity to the S10 and spc operons of *E.coli.*- Proc. nat. Acad. Sci. USA **83**: 6030-6034, 1986.

Wochok, Z.S., Sussex, I.M.: Redetermination of cultured root tips to leafy shoots in *Selaginella willdenovii*. - Plant Sci. Lett. **6**: 185-192, 1976.

Zhang, Y., Chen, M., Chou, B.B., Jermiin, L.S., Larkum, A.W.: Evolution of the inner light harvesting antenna protein family of cyanobacteria, algae and plants. - J. mol. Evol. **64**: 321-331, 2007.