Expression of two cloned mRNA sequences during development and germination of spores of the sensitive fern, Onoclea sensibilis L.

V. Raghavan 1* and Joseph C. Kamalay2

1 Department of Plant Biology and 2 Department of Agronomy, The Ohio State University, Columbus, OH 43210, USA

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Abstract. A library of complementary DNA (cDNA) clones has been prepared from poly(A)+RNA of spores of the sensitive fern, Onoclea sensibilis L. By differential hybridization with labeled probes made to poly(A)+RNA of spores, gametophytes and leaves, two spore-specific clones (pOSS68 and pOSS194) were selected and characterized. Northern blot analysis showed that RNA sequences homologous to the two cDNA clones first appear in the post-meiotic spore and increase in abundance during spore maturity. Both RNA sequences decay during photoinduced germination of the spores and do not reappear in the gametophytes. In spores imbibed in the dark under conditions which do not favor germination, no significant decrease in pOSS194-mRNA abundance is noted. In contrast, the decrease in pOSS68 mRNA in dark-imbibed spores parallels that observed in photoinduced spores. The predicted amino-acid sequence of pOSS 194 has a striking similarity to the early light-inducible proteins expressed during the greening of etiolated pea and barley seedlings, whereas that of pOSS68 shows some homology to proteins encoded by late-embryogenesis-abundant mRNAs of angiosperm embryos.

Key words: Onoclea (cDNA library) - Light and development - Protein (late embryogenesis abundant) - Protein (early light-inducible) - Spore-specific mRNA

Introduction

In homosporous ferns, germination of a uninucleate spore initiates the growth of the gametophyte leading to the production of sex organs, antheridia and archegonia. Included in the progression of events of spore germination are migration of organelles, cell division and differentiation of distinct cell types such as the rhizoid and the protonemal cell. As in some angiosperm seeds, germination of spores of several ferns is potentiated by red light and reversed by far-red light, implicating phytochrome control. Also analogous to seeds, spore germination in some ferns is induced by gibberellic acid (for review, see Raghavan 1989, 1992).

A previous work (Raghavan 1987) showed that spores of the sensitive fern, Onoclea sensibilis contain stored mRNAs, the amounts of which decrease during red light-induced germination. Recently, it has also been found that under conditions which do not promote dark germination, both dark-imbibed and photoinduced spores display, in vivo, similar protein-synthetic profiles during the first 48 h after sowing. Of interest was the finding that except for three new protein bands, all in-vivo-synthesized proteins correspond to those synthesized in vitro by poly(A)+RNA isolated from dormant spores (Raghavan 1991). From these results it appears that stored mRNAs are functionally active during imbibition and germination of spores and that photoinduction leads to the synthesis of proteins encoded on some rare, stored mRNAs.

To isolate these mRNAs and to analyze the molecular biology of spore development and germination, we have constructed a cDNA library made to poly(A)+RNA of spores of O. sensibilis and have identified by differential screening several spore-specific sequences. Of the two cDNA clones selected for further study, amino-acid sequences of one show similarity to the early light-inducible proteins (ELIP) of pea and barley seedlings and those of the other show similarity to the late embryogenesis abundant (LEA) sequences of angiosperm embryos. The results of these studies are reported in this paper.

Material and methods

Plant material. Fertile fronds of O. sensibilis L. were supplied by Dr. Todd J. Cooke, University of Maryland. They were wrapped in a
polyethylene bag and stored at −20°C for approx. one year before use. Sporangia were freed from fronds and gently crushed with the forefinger to liberate the spores. The latter were separated from sporangial walls and other debris by sieving successively through 200- and 100-μm-wide U.S. standard sieves. These spores were used without further treatment for making a cDNA library.

In other experiments, spores were harvested at 12, 24, 36, 48, 72 and 96 h following dark-imbition or photoinduction. For this purpose, spores were sterilized by swirling them for 3 min in a commercial bleach diluted to 0.025% NaOCl and washed several times with sterile distilled water. They were transferred to a sterile Petri dish and dried overnight in vacuo. Batches of approx. 25 mg of sterilized spores were placed in 10-cm-diameter Petri dishes containing 20 ml sterile Knop’s mineral-salt medium (Raghavan 1965) and the dishes were immediately kept in a dark incubator at 25°C or exposed to red light (67.4 μW·cm−2) obtained by filtering light from three 15-W cool-white fluorescent tubes through a 3-mm-thick red Plexiglas (No. 2444; Rohm & Haas, Philadelphia, Pa., USA). Samples of spores were harvested by suction on a filter paper placed on a Millipore filter unit and washed with sterile water. To collect gametophytes of different ages (1, 2, 5, 10 and 20 d old), spores allowed to germinate in red light for 4 d were transferred to paper placed on a Millipore filter unit and washed with sterile water.

Fronds on which the pinnae had fully expanded and sori of different ages were collected from plants growing at the Gahanna Woods State Nature Preserve, Columbus, Ohio, USA. Samples of sori and sporangia and spores separated from them were immediately fixed in formalin-acetic acid-alcohol (formalin 5 ml, glacial acetic acid 5 ml, 50% ethanol 90 ml) to monitor the stages of sporogenesis by acetocarmine-squashing and by sectioning. For the latter purpose, the tissues were dehydrated successively in 70, 90 and 100% ethanol, n-propanol and n-butanol and embedded in glycol methacrylate (Feder and O’Brien 1968). Sections, 5–7 μm thick, cut with a steel knife fixed to a rotary microtome, were stained with 0.1% Toluidine Blue O. Slides were air-dried and mounted in Euparal for microscopic examination.

**Extraction of RNA and isolation of poly(A)+ RNA.** Spores freshly separated from the sporangia and freshly collected gametophytes, leaves and sori were used for RNA extraction. In the protocol followed here, 0.2- to 0.5-g portions of the material were ground in a mortar or in a glass homogenizer with 10 ml of a buffer containing 500 mM Tris (pH 8.0), 2.5 M sodium perchlorate, 2.5% sodium dodecyl sulfate (SDS), 25 mM EDTA, 25 mM NaCl, 2.5 mM sodium bisulphite and 8% insoluble polyvinyl pyrollidone (PVP). Following centrifugation at 11500 × g for 30 min at 4°C, the upper aqueous layer was collected and extracted once with phenol and twice with phenol-chloroform (1:1, v/v), separating the aqueous layer was collected and extracted once with phenol and once at 50°C for 1 h with 2× SSC containing 0.1% SDS and finally once for 10 min at 25°C with 2× SSC. Dried filters were exposed to Kodak X-Omat X-ray film with an intensifying screen at −70°C. The positive colonies were further screened using 32P-random primer-labeled ss-cDNA probes prepared to poly(A)+ RNA of dormant spores (Sambrook et al. 1989), using [32P]dCTP (111 TBq·nmol−1; ICN Radiochemicals, Irvine, Calif., USA). After hybridization at 42°C for 24 h, the filters were washed once at 25°C for 15 min, twice at 50°C for 30 min each and once at 50°C for 1 h with 2× SSC containing 0.1% SDS and finally once for 10 min at 25°C with 2× SSC. Dried filters were exposed to Kodak X-Omat X-ray film with an intensifying screen at −70°C. The positive colonies were further screened using 32P-random primer-labeled ss-cDNA probes prepared to poly(A)+ RNA of dormant spores, white-light-grown gametophytes and leaves. Bacterial colonies which showed a greater intensity of hybridization with the probe prepared from spores than with the other two probes were selected for further analysis.

Small amounts of plasmid DNA were isolated by the method of Holmes and Quigley (1981). Large amounts were prepared from 1-L cultures by the alkaline-lysis procedure (Sambrook et al. 1989), followed by equilibrium banding in a CsCl-ethidium bromide gradient. The authenticity of cDNA inserts was confirmed by EcoRI digestion of the plasmid DNA, followed by agarose-gel electrophoresis.

**Northern blot analysis.** Total RNA (10 μg) was electrophoresed on 1% agarose gels containing 6% formaldehyde and the fragments were transferred to Nitran filters (Schleicher & Schuell) by capillary blotting using 10× SSC as the solvent. The filters were baked as before and were prehybridized for 12 h at 42°C in the same solution as for differential screening except that 30 μl of herring-sperm DNA (10 mg·ml−1) was substituted for poly(A). Hybridization was carried out in the same buffer containing approx. 5 × 106 cpm·ml−1 32P-labeled cDNA insert for 24 h at 42°C. To prepare probes of cDNA inserts, plasmid DNA was restricted with EcoRI and electrophoresed on 1% agarose gel. The insert was eluted from the gel, purified by passing through an Elutip-D column (Schleicher & Schuell) and concentrated by ethanol-precipitation. DNA was labeled with [32P]dCTP by the method of Feinberg and Vogelstein (1983) using random hexamer primers (Pharmacia) and Klenow fragment (BRL, Gaithersburg, Md., USA). After hybridization, the filters were washed, dried and autoradiographed as described before.