Differential Localization of Gossypitrin and Herbacitrin in *Equisetum* Sporelings

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The senior author noticed that the inside of the young sporeling of *Equisetum arvense* turned yellow when immersed in an alkaline solution, and that the coloration was always restricted to the basal cell of the young protonema. The colored matter was extracted naturally into the alkaline medium, and the crude extract was examined with a spectrophotometer. As a result, it was tentatively considered to contain a kind of flavonoid. Later, it was chemically purified by the junior author and was identified to consist of two substances, gossypitrin and herbacitrin, as reported in this paper.

Material and method

Fresh spores of *Equisetum arvense* L. were collected in Yamagata as the material and were stored in a refrigerator under dried conditions at about -5°C . The spores were taken out according to demand of the experiments, washed by shaking in distilled water for about 1 minute, then they were sown in Knop's medium diluted to 1/5 of the standard concentration, contained in petri dishes. The depth of the culture medium was about 3 mm, the density of the spores was about 5 individuals for 1 mm^2 , temperature was about 20°C , under constant illumination of white light at 1000 lux by use of 40 w fluorescent tubes. Under these conditions, the spores sank to the bottom of the dish and germinated well. 0.1 M KOH water solution was prepared for the test of coloration.

For chemical examination, the dried spores (500 g) were extracted for two weeks with acetone at room temperature, then the solution (A) and the residue were separated. The residue was then extracted with methanol (Soxhlet) for 48 hr, and the methanol solution was separated from the residue (B). The methanolic extracts were concentrated to a small volume, to which was added ether. The etherial layer (C) was separated from the insoluble brown solid (D) by decantation. Finally the brown solid (D) was analyzed into further constituents in paper chromatography by use of Toyo Roshi No. 5 paper in $n\text{-BuOH-AcOH-H}_2\text{O}$ (4 : 1 : 2), and the constituents were examined of their fluorescence and were detected with $\text{Pb}(\text{OAc})_2$.

As the second method of chemical examination, the above fraction (D) was column-chromatographed on a cellulose microcrystalline (Merck, Art 2331) using 0.3% HOAc-sat. $\text{H}_2\text{O} \cdot \text{AcOEt}$ as the solvent. In both methods, two pure substances

were obtained, 1.45 g (0.29%) (E) and 0.55 g (0.11%) (F) respectively, and they were examined of their m.p., UV spectra in 95% ethanol, R_f value, and IR spectra.

The substances purified as above were compared with authentic samples of gossypitrin and herbacitrin. UV spectra were measured using a Hitachi EPS-3T spectrometer. IR spectra were determined on KBr disks using a Hitachi 215 grating infrared spectrometer.

Results

Spores washed with distilled water were mounted on a glass slide with a drop of distilled water, observed under a microscope, then a drop of 0.1 M KOH solution was added. Suddenly the

cytoplasm of the spore turned yellow (Figs. 1A, B), then the coloration moved to the medium around the spore, and the spore lost the yellow color. This shows that the fresh spore contained a substance or substances colored yellow with alkali and the substance(s) naturally diffused out into the medium.

Fresh spores well germinated in Knop's medium in 10 hr, and they were divided into two cells, the rhizoid and the initial cell of protonema. At this stage, by addition of KOH solution, the yellow coloration appeared in the initial cell of protonema uniformly (Figs. 1C, D). Next 10 hr later, the initial cell of protonema was divided into two, one of which began to elongate as the apical cell, but the other remained in quiescence as the basal cell. At this stage, the coloration by KOH occurred only in the basal cell. After the protonema developed to 3- and 4-cell stages, also the coloration with KOH was observed only in the basal cell (Figs. 2A, B, C, D). In later stages, gradually, even the basal cell lost this special characteristic.

Fig. 1. Spores and sporelings of *Equisetum arvense*. A, fresh spores. B, the same with KOH displaying yellow coloration which shows presence of gossypitrin and herbacitrin. C, sporelings consisting of a rhizoid and the initial cell of protonema. D, the same with KOH. Scales represent 50 μ m.

Fresh spores were centrifuged at 50,000 g for 3 minutes, which resulted in stratification of the intracellular materials. Lipid layer came to the centripetal end, then the layer of chloroplasts involving the nucleus, followed by the hyaline layer of cytoplasm, and finally mitochondria gathered at the centrifugal. Examination of the centrifuged spores with KOH solution revealed that the yellow coloration appeared most remarkably in the centripetal region containing lipid and chloroplasts.

Fresh spores (0.1 g), after being washed repeatedly with distilled water, were immersed in 20 ml of 0.1 M KOH solution, and the yellow-colored matter was extracted into the medium. The medium containing the colored matter was put into quartz cells, and examined by use of a spectrophotometer. The UV spectrum of this solution showed a characteristic absorption peak at around 260 nm. The same measurements were also carried out with pure gossypitrin and herbacitrin separately dissolved in 0.1 M KOH solution. It was found that the absorption peaks at around 260 nm of the both compounds were quite similar to the spore.

The final fraction (D), obtained by extraction with methanol of the fresh spores, was examined by the junior author. The components (E) and (F) were identified as gossypitrin and herbacitrin respectively by comparing their m.p., IR (Fig. 3) and UV spectra with authentic samples. $UV_{\lambda_{max}}^{EtOH}$ nm (log ϵ): (E) 261 (4.14), 286 (3.98), 347 (3.93), 388 (3.96); (F) 226 (2.76), 252 (2.75), 273 (2.80), 335 (2.68), 277 (2.71); m.p. (dp): 225°C, (F) 233–236°C; RF value: (E) 0.34, (F) 0.49.

Discussion

Considered from the above experiments, it is clear that the spore of *Equisetum arvense* contains gossypitrin and herbacitrin. Therefore, the yellow coloration with KOH may be attributed to these substances.

They are uniformly distributed in the fresh spore. Later, after germination, they are contained mainly in the initial cell of protonema, and after that cell was divided into two, they are contained mainly in the basal cell, but little in the apical cell. As to this phenomenon, three possibilities are considered. The first is that gossypitrin

Fig. 2. A, C, two-celled protonema. B, D, the same with KOH. Coloration is seen only in the basal cell. Scales represent 30 μ m.

Fig. 3. IR absorption spectra of two substances, gossypitrin and herbacitrin, taken out of the *Equisetum* spore.

and herbacitrin were translocated from the apical to the basal cell. The second is that they were changed to other substances in the apical, but remained unchanged in the basal cell. The third is that they diffused out of the apical cell into the medium, but did not in the basal cell. At present, it is not clear which possibility is the case. In addition, there is the question whether the apicobasally differential localization of these substances is a cause or a result of determination of the apico-basal polarity of the developing sporeling.

Gossypitrin was first taken out in 1916 from the flower of *Gossypium* by Parkin. Later it was obtained from *Chrysanthemum* flower by Geissman and Steelink (1957), and from *Equisetum* strobili including spores and other tissues by Nakabayashi (1957) and by Kutney and Hall (1971). However, in their reports, it is not clear whether it was contained in spore or in other tissues. In the present research it was verified that it was contained in spore, but not or hardly in other tissues. In addition, it was also clarified that the same spore contained herbacitrin. According to Nakazawa (1978, 1979), the rhizoid development of *Fucus* eggs and early development of sea urchin eggs are inhibited by gossypitrin at 10^{-3} M in concentration. This implies that gossypitrin takes part in morphogenesis of *Equisetum* sporelings for differential regulation of cellular activities.

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Summary

When the spore of *Equisetum arvense* is immersed in 0.1 M KOH solution, the cytoplasm is colored yellow, and the colored matter is naturally extracted into the medium. After germination, the same coloration with KOH takes place in the basal cell of protonema but not in the apical cell. The colored matter was taken out from the spore with acetone followed by methanol and ether, then it was analyzed by paper-chromatography and by column-chromatography. The analyzed components were separately examined by use of UV absorption and IR absorption spectrometries. As a result, the colored matter was identified to be consisting of gossypitrin and herbacitrin. Role of these two substances in the cell of *Equisetum* is not clear, while presumed to take part in regulation of morphogenesis.

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