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## Auxin modulation of differentiation-specific polypeptides in the protonema cultures of the wild strain and auxin mutants of *Funaria hygrometrica*

Maintenance of chloronema state and the onset of caulonema state and of caulonema differentiation in the protonema of *Funaria hygrometrica* are regulated by endogenous cyclic 3',5'-AMP and indole-3-acetic acid (IAA), respectively<sup>1-6</sup>. The role of these growth regulators is further controlled through an inverse relationship between the activities of cyclic nucleotide phosphodiesterase (cNPD) and IAA oxidase in the protonema<sup>7</sup>. One of the significant subcellular changes during the transition from chloronema to caulonema involves a change in the shape, structure and distribution of chloroplasts<sup>3</sup>. There are some reports on the detection of caulonema-specific proteins in the whole tissue protonema extracts of *Funaria*<sup>8-10</sup>. In the present work, a comparative analysis of the age-dependent and auxin-triggered changes in the polypeptide profile in the soluble fractions of whole tissue extracts and chloroplast fraction of the wild strain and two auxin mutants, has been undertaken in order to detect the possible changes in the location and distribution of auxin-induced differentiation-specific proteins, between chloroplasts and extrachloroplast cytoplasm.

Mutants were isolated at the Botanisches Institut, University of Heidelberg (Germany) by UV irradiation of proto-

plasts, as described earlier<sup>11</sup>. Cultures raised on Knop's nutrient medium solidified with 2% agar, were kept under continuous illumination (4.32 Watts m<sup>-2</sup>) at 25 ± 2°C and maintained by regular subculturing after every 8-10 days. The data presented were recorded 48 and 120 h after subjecting the 7-day-old protonema cultures to various treatments. For SDS PAGE of polypeptides, protonema tissue was homogenized in an ice-cold grinding medium (30 mM Tris-MES, pH 8, containing 0.4 M sucrose, 10 mM KCl, 1 mM EDTA.Na<sub>2</sub>, 0.1 mM MgCl<sub>2</sub>, 1 mM β-mercaptoethanol and 1.5 mM phenylmethylsulphonyl fluoride). The supernatant obtained after centrifugation at 5500 g was used to precipitate soluble proteins by mixing with 8 volumes of ice-cold acetone for 3 h at -20°C. Chloroplasts were isolated by homogenizing protonema (50 mg fw) in 1 ml of buffer (0.33 M sorbitol, 0.2 mM MgCl<sub>2</sub> and 20 mM MES, brought to pH 6.5 with Tris)<sup>12</sup>. The resultant slurry was recentrifuged at 500 g for 1 min. The supernatant was recentrifuged at 2700 g for 1 min to get crude chloroplast fractions. The pellet thus obtained was resuspended in a cation-free medium (0.33 M sorbitol, brought to pH 7.5 using Tris), for purification by repeated centrifugation (3 times) at 2700 g for 1 min each.

This procedure resulted in a preparation consisting of more than 90% intact and active chloroplasts, as determined by their activity in Hill's reaction. For precipitating the chloroplast proteins, the chloroplast pellet was resuspended in 0.33 M sorbitol (pH 7.5) followed by acetone precipitation. The precipitated proteins were pelleted by recentrifugation at 5500 g for 10 min. The pellet was air-dried and redissolved in Laemmli buffer (0.06 M Tris, 2% glycerol and 5% β-mercaptoethanol). After removing an aliquot for the estimation of total protein content by Bradford assay<sup>13</sup>, the remaining protein sample was subjected to SDS treatment<sup>9</sup>. About 20 µg protein was loaded in each well of the 1.5 mm thick, 12.5% polyacrylamide gel, from the whole tissue samples and 15 µg from each of the chloroplast preparations. The polypeptide profiles were visualized by silver staining<sup>14</sup>.

Under the above-mentioned culture conditions, wild strain protonema of *Funaria* exhibits normal growth and development in terms of chloronema formation, followed by spontaneous caulonema differentiation after 8 days of growth on hormone-free Knop's medium (basal medium). Wild strain protonema exhibits further promotion of caulonema differentiation by IAA (50 µM) treatment.



Mutant 87.13 (auxin-sensitive) remains as chloronema on basal medium. It responds to IAA treatment by forming caulonema. Mutant 86.1 is insensitive to auxin treatment and remains as chloronema (Figure 1). A comparison of the whole tissue polypeptide patterns in the chloronema cultures (7-day-old) of the three strains shows remarkable differences both in high (78 kDa) and low molecular weight (< 29 kDa) polypeptides (Figure 2 *a-c*; lane 1). Mutant 86.1 (auxin-insensitive) shows maximum divergence from the wild type with regard to polypeptide patterns in the whole tissue extracts (Figure 2 *a, c*; compare lane 1).

Spontaneous induction of caulonema in the chloronema cultures of wild strain is accompanied with the suppression of at least five polypeptides in the high molecular weight range (78 kDa; Figure 2 *a*; lane 2). Subjecting the wild type cultures to IAA treatment results in suppression of these polypeptides (Figure 2 *a*; lane 3) accompanying enhancement of caulonema differentiation. These high molecular weight proteins tend to get expressed in the auxin-sensitive mutant 87.13 (Figure 2 *b*; lane 3) and their expression is most pronounced and unaltered by auxin treatment in the mutant 86.1 (Figure 2 *c*; lane 3). The trend con-

tinues in the extracts of older protonema (Figure 2 *a-c*; lanes 4, 5). In the low molecular weight range, the polypeptide patterns exhibit a reverse trend as compared to high molecular weight polypeptides. Thus, the expression of polypeptides with < 29 kDa is least in the auxin-insensitive mutant (86.1). In the wild strain and auxin-sensitive mutant (87.13), their expression is better than in the auxin-insensitive mutant. There is practically no difference in the accumulation of chloroplast polypeptides with age and auxin treatment in the wild strain preparations (Figure 2 *d*; lanes 1-5). In the auxin-sensitive mutant (87.13), IAA

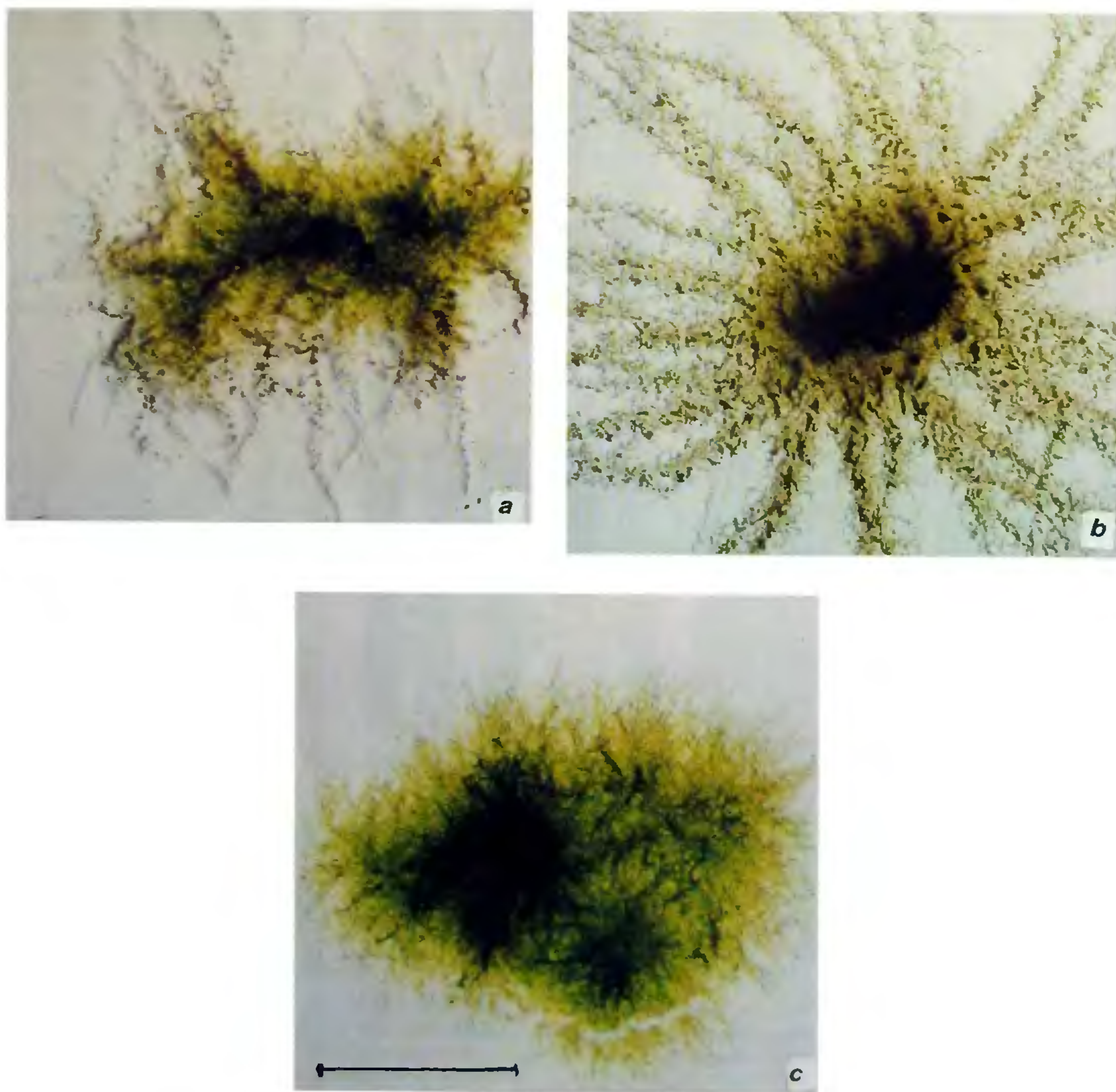


Figure 1. Protonema cultures of *a*, the wild strain; *b*, auxin-sensitive mutant 87.13; and *c*, auxin-insensitive mutant 86.1. The cultures are 7-day-old and are fully chloronematic at this stage of growth. Bar = 1 mm.

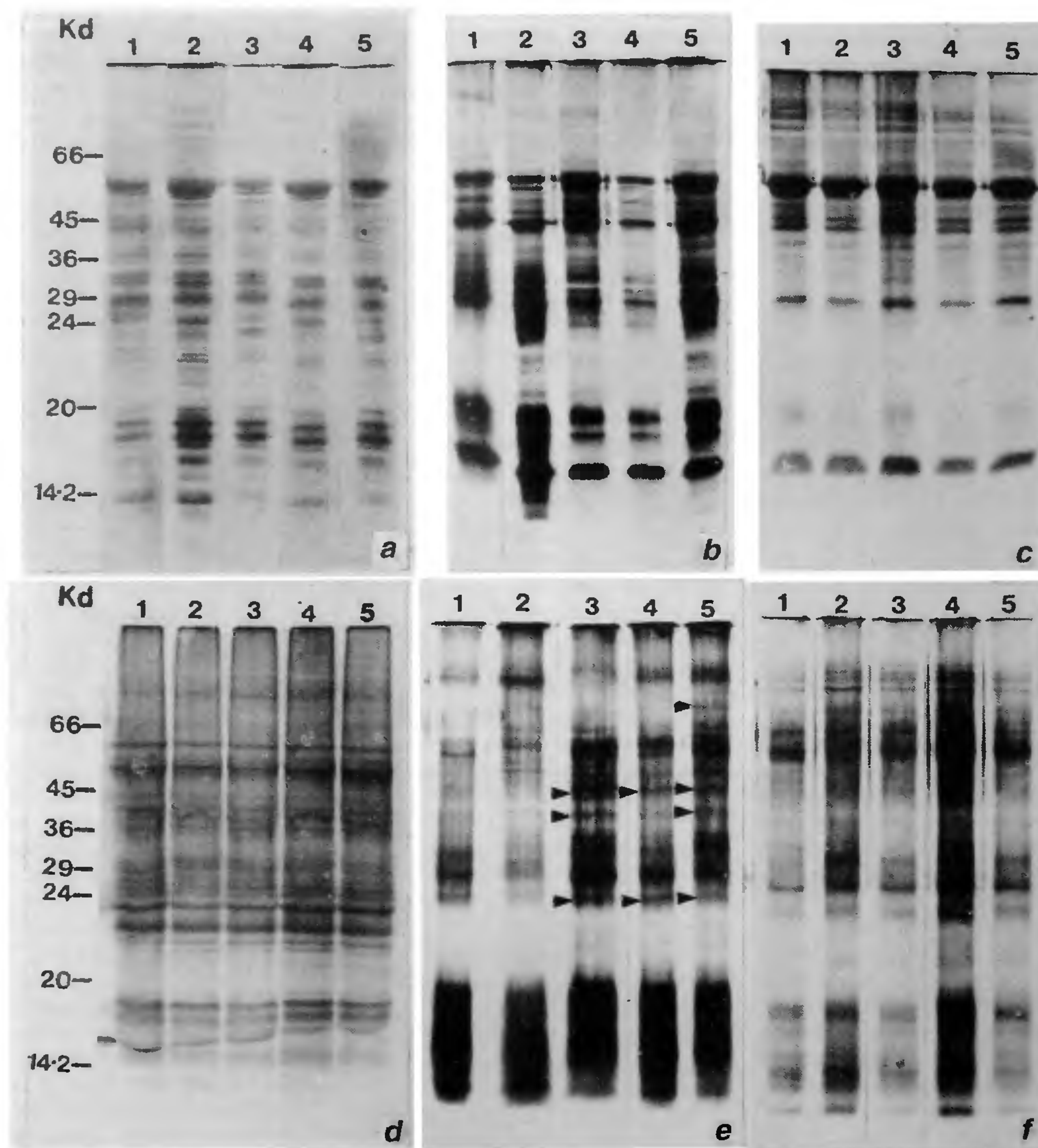


treatment of protonema brings about the expression of certain polypeptides in the chloroplasts (shown with arrowhead in Figure 2e), accompanying caulonema differentiation. The accumulation of polypeptides in a similar molecular weight range in chloroplast preparations of auxin-insensitive mutant is, however, not affected in a similar fashion. This is in

congruence with the insensitivity of this mutant to auxin treatment with regard to protonema differentiation. A set of high molecular weight proteins is, however, clearly seen in the chloroplast preparations of this mutant, irrespective of age and auxin treatment (shown by arrowhead in Figure 2f).

A set of high molecular weight pro-

teins (78 kDa) seem to be chloronema-specific and their expression is not altered by auxin treatment. The expression of some low molecular weight proteins (< 29 kDa) appears to accompany auxin-induced caulonema differentiation in the wild strain and auxin-sensitive mutant. Accumulation of certain chloroplast polypeptides in the molecular



**Figure 2.** Age- and auxin- ( $10^{-5}$  M IAA) dependent changes in polypeptide patterns of the 5500 g whole tissue soluble fractions and chloroplast fractions, as analysed by vertical gel electrophoresis using 12.5% SDS PAGE gel. *a*, Whole tissue soluble fraction polypeptide profile in wild strain; *b*, auxin-sensitive mutant 87.13; and *c*, auxin-insensitive mutant 86.1. *d*, Chloroplast polypeptide profile in wild strain; *e*, mutant 87.13; and *f*, mutant 86.1. Lane 1, Polypeptide profile of 7-day-old protonema cultures raised on basal (hormone-free) medium; lane 2, results from 7-day-old cultures further grown for 48 h in control ( $-$  IAA) conditions; lane 3, polypeptide profile from cultures as in lane 2 but grown in the presence of IAA ( $10^{-5}$  M); lane 4, 7-day-old cultures further grown for 120 h in control ( $-$  IAA) condition; lane 5, polypeptide profile after IAA treatment for 120 h. Significant changes have been shown with arrowheads.



weight range of 24–45 kDa also occurs in response to auxin treatment, accompanying caulonema differentiation in the auxin-sensitive mutant. This indicates that probably these chloroplast polypeptides accumulate during auxin-induced protonema differentiation. The fact that two of these polypeptides (Figure 2e; lane 4, shown with arrowhead) also appear in untreated cultures at a later stage of growth, is in agreement with some degree of caulonema differentiation at this stage<sup>15</sup>, indicating that these chloroplast proteins are caulonema-specific and their accumulation is promoted by auxin treatment. Likewise, certain high molecular weight proteins expressed in chloroplast preparation of the auxin-insensitive mutant (marked with arrowhead) indicate their preferential localization in the chloroplasts. Thus, some of the auxin-regulated polypeptides in the whole tissue extracts seem to be of chloroplast origin. Auxins have been found to induce the synthesis of new specific proteins in many higher plant tissues. Transcriptional regulation of auxin-responsive genes is well established for soybean genes, whereby activated as well as res-

tricted genes are found with respect to specific tissues or organs<sup>3,16</sup>. Therefore, it can be expected that in moss protonema, which has a very specific auxin-regulated protonema development, differentiation-specific proteins are detected.

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## Screening chemical hybridizing agents for development of hybrid wheat

The wheat (*Triticum* spp.) production during 1998–99 was around 73.2 million tons from 26.2 million hectare. The north-western plain zone (NWPZ) that sustains the food security system accounts for 36 per cent of cropping area, i.e. 9.5 million hectare. Here the per hectare productivity has become static and genetic gain of just 1% yield per year is being achieved by conventional plant breeding efforts. Therefore a need has arisen to look into alternate plant breeding approaches to break the present deadlock of stagnant per hectare productivity. The heterotic advantage that is being harnessed in maize, sunflower, sorghum and rice offers an exciting opportunity to increase wheat yield further. Wheat being a self-pollinated crop, an allo-hexaploid and a natural hybrid has several inherent limitations in heterosis breeding. But the new chemical hybridizing agent (CHA) mole-

cules offer opportunities to induce selective male sterility, promote out-crossing and develop viable hybrids.

Efforts to induce male sterility in wheat with chemicals were initiated in the early fifties with little success. In the early seventies, a number of private concerns developed and tested chemicals like Dalpon, DPR 3778, Estrone, Gibberellic acid, Hybrex, LY 195259, Mendok, RH series, WL 84811, etc.<sup>1</sup>. These chemicals can be classified as growth regulators, growth retardants and analogues and exhibited partial to near-complete male sterility along with variable damage to plant vegetative growth and flowering. The discovery of CHA or gametocides provided a new way for inducing male sterility, thereby enhancing the process of selecting parental lines leading to commercial heterosis. In the last 10 years significant efforts have been made for

commercial exploitation of hybrid wheat through the use of gametocide. In India research efforts on hybrid wheat were initiated in the mid-sixties primarily using a three-line approach; however, no significant results were obtained. Since 1995, we have re-addressed the issue of hybrid wheat by synthesizing 41 different molecules that were considered as potential CHA candidates. The objective was to identify a suitable CHA to induce perfect male sterility at a suitable dose and crop growth stage.

Two spring wheat varieties, viz. WH 542 (JUP/BJY//URES) and PBW 343 (ND/VG 7944//KAL//BB/3/YACO'S/4/VEE # 5'S') were grown in a plot in four rows of two metres each at the Directorate of Wheat Research, Karnal. Twenty-three molecules at different doses were evaluated over WH 542 and PBW 343 for their efficacy to cause selective male sterility.