ISOENZYME ANALYSIS OF CYTOKININ SENSITIVE MUTANTS OF THE MOSS *

PHYSCOMITRELLA PATENS*

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(Received February 6th, 1989)
(Revision received May 29th, 1989)
(Accepted May 29th, 1989)

Two mutants of the moss Physcomitrella patens, severely impeded in bud formation (PC22 and P24), and the fusion product PC22(+)P24 were compared to the wild type. We investigated their cytokinin sensitivity and the isoenzyme patterns of their esterases, malate dehydrogenases and peroxidases at distinct states of differentiation. None of these enzymes appeared to be specific to the differentiation process of budding. In the physiological test the fusion product behaves like the wild type and differs from the two mutants. The isoenzyme data suggest that PC22(+)P24 contains genetic information from both mutant strains. Using the DNA-specific DAPI stain we could rule out PC22(+)P24 being a dicaryotic plant.

Key words: cytokinin; esterases; malate dehydrogenases; moss mutants; Physcomitrella patens; peroxidases

Introduction

The moss Physcomitrella patens is an excellent system for investigating the effects of growth regulators, such as cytokinins, on differentiation processes. (i) It exhibits a sequence of well defined differentiation states: chloronema, caulonema, buds and leafy gametophores. (ii) Large numbers of intact and well differentiated plants can easily be cultivated axenically. The bud inducing effect of exogeneous cytokinins is well documented for a variety of moss protomenclata [1 — 4].

To study the mechanism of phytohormone action in mosses developmentally abnormal mutants are increasingly used as such mutants are easily obtained from the haploid spores or the well regenerating haploid protoplasts [5—8]. Most of these mutants are infertile, precluding classical genetic analyses. Recently, to circumvent this problem a number of groups have begun to fuse mutant protoplasts and analyse the fusion products [9—13].

We induced mutants of P. patens by X-ray treatment of the haploid spores. Some of the mutants obtained are sensitive to exogeneous cytokinins [14]. Hansen et al. [13] electrofused single protoplasts of two of these mutants, which either do not form buds without exogeneous cytokinins (P24) or at the most only a few after a long cultivation period (PC22). One resulting fusant, PC22(+)P24, did form buds spontaneously without exogeneous cytokinins and even produced leafy gametophores, indicating complementation in the fusion product.

Although there is a vast amount of detailed information about somatic hybrids in higher plants [15,16] and ongoing studies on somatic hybridization of haploid protoplasts of higher plants [17,18] there are so far no reports about either (i) the biochemical characterization of fusion products in mosses or (ii) the fate of

Abbreviations: DAPI, 4'6-diamidino-2-phenylindole; EST, esterase(s); fAde, isopentenyladenine; MDH, malate dehydrogenase(s); PAA, polyacrylamide; FX, peroxidase(s); WT, wild type.

*This article is based in part on a doctoral study of R.R. at the University of Hamburg, Faculty of Biology.

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0168-9452/89/$03.50 © 1989 Elsevier Scientific Publishers Ireland Ltd.
Printed and Published in Ireland
nuclear genes in fusion products obtained by one-to-one electrofusion of defined protoplasts pairs. We describe here physiological and isoenzyme analyses to further characterize a wild type strain of the moss, *P. patens*, two developmental mutants, derived from it, and a fusion product of the two mutant strains, in order (i) to obtain information about the fate of nuclear genes in this particular fusant originated by one-to-one protoplast fusion of defined haploid moss protoplasts, and (ii) to establish a model system for further investigating the mechanism of cytokinin-induced differentiation in mosses.

**Materials and Methods**

**Plant material**

The wild type (WT) is described in Reski and Abel [4]. The two mutants (PC22 and P24) were obtained from X-ray treated spores of this strain individually cultured in unsupplemented Knop medium [19].

PC22 is capable of developing a few buds after a long cultivation period without exogenously applied cytokinin. These buds never differentiate into leafy gametophores as they show callus-like growth and finally necrosis. Furthermore, PC22 is characterized by one large chloroplast per cell [14].

P24 never develops buds when cultivated on unsupplemented Knop medium. The phenotype of P24 exhibits shorter chloronema cells than the wild type, and some of them are swollen. However, they have wild type-like chloroplasts.

After one-to-one electrofusion of single mutant protoplasts, a fusion product, PC22(+)+P24, could be regenerated that does form buds and even leafy gametophores on Knop medium [13]. Furthermore, cell shape as well as chloroplast shape and number are indistinguishable from the wild type.

**Culture conditions and isoenzyme sampling**

Protonemata were cultured axenically in an agitated liquid Knop medium as described [4]. On the 12th day isopentenyladenine (i6Ade) (Serva) from a methanolic stock solution was added to selected flasks to a final concentration of $10^{-5}$ M. As a degree of cytokinin sensitivity we measured the time in days after that protonemata exhibited cytokinin induced buds of at least three cells.

Even under constant growth conditions isoenzyme patterns may vary in the course of plant growth and development (e.g., Refs. 20—22). We therefore prepared samples for isoenzyme analysis at distinct differentiation states during the course of development of all four genotypes.

For this purpose we started isoenzyme analysis at day 12 where the wild type, the two mutants and the fusant were differentiated solely into chloronema and caulonema. At all subsequent days, when the wild type, the fusion product or one of the mutants exhibited cytokinin induced budding we took samples of the four genotypes from cytokinin treated as well as from untreated control plants showing no budding. In this way we got reproducible banding patterns most probably specific for the distinct states of differentiation.

**DAPI-staining**

Protonemata were fixed for 30 min in 4% glutardialdehyde, chlorophyll was extracted with methanol, the protonemata were stained with 2 μg/ml 4',6-diamidino-2-phenylindole (DAPI) (Serva) for 30 min and washed in distilled water for another 30 min.

**Native PAA gel-electrophoresis and specific staining**

**Enzyme extraction.** Protonemata (0.2 g) were ground to a fine powder in liquid nitrogen and thawed at 4°C in 1 ml of the extraction buffer (0.05 M phosphate buffer, pH 8.0). The crude extracts were centrifuged for 20 min at 10 000 rev./min at 4°C. Total protein concentration was calculated according to Bradford [23] and was adjusted to 1.1 μg/μl. After adding 10% (w/v) glycerol the supernatants were electrophoresed either immediately (EST, MDH) or after overnight storing at 4°C (PX).

**Electrophoretic procedures.** Electrophoreses were performed in vertical discontin-
uous polyacrylamide gels at 4°C. The gels for EST as well as those for MDH were composed of a 10% acrylamide Tris-citrate running gel at pH 8.9 and a 4% acrylamide Tris-citrate stacking gel at pH 6.8, whereas gels for PX were composed of a 7% acrylamide Tris-HCl running gel at pH 8.8 and a 3% acrylamide Tris-HCl stacking gel at pH 6.7.

After preelectrophoresis of the gels for 30 min at 3 V cm⁻¹ the buffers were changed, the sample extracts (100 µl for EST and PX, 40 µl for MDH) were loaded onto the gel, and electrophoresed for 4 h at 10 V cm⁻¹ (EST, MDH) or 5 h at 15 V cm⁻¹ (PX). As we analysed different physiological states of the four genotypes we electrographed equal amounts of total protein and not equal amounts of enzyme activity. In control experiments we could not detect additional isoenzyme bands even if a five-fold quantity of total protein was loaded onto the gels.

Enzyme-specific stains. Gels were stained for EST following the method of Ainsworth et al. [24], for MDH as described by Arus and Orton [25] and for PX according to the method of Scandalios [26].

Results

Cytokinin sensitivity

In this paper we take as model for cytokinin sensitivity the period of time, which 10⁻⁶ M i⁶Ade needs to induce buds of at least three cells on 12-day-old protonemata. WT as well as PC22 (+)P24 protonemata exhibited budding 3 days after cytokinin treatment, whereas untreated control plants did not show budding. PC22 protonemata reacted with budding 7 days after cytokinin treatment. i⁶Ade (10⁻⁵ M) applied to 12-day-old P24 protonemata-induced buds after 21 days (Fig. 1). The same length of cytokinin treatment leads to necrosis in the WT, the mutant PC22 and the fusant PC22(+)P24.

DAPI-staining

To exclude the possibility that the fusant PC22(+)P24 was a dicaryotic plant, we stained protonemata of it with the DNA-specific fluoro-

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Fig. 1. Test for cytokinin sensitivity: Time in days (blank areas) 10⁻⁴ M i⁶Ade needs to induce buds of at least three cells on 12-day-old (hatched areas) moss protonemata.

Isoenzyme analyses

Proteins of defined states of differentiation were separated on native PAA-gels and specifically stained for EST, MDH and PX. Examples of such gels are presented in Figs. 3—5.

EST. The data of a number of experiments are presented in Fig. 6. We could detect nine different esterase bands. Six of them (EST-1’, EST-1, EST-2, EST-5, EST-6, EST-7) most probably are expressed constitutively in every tissue investigated.

EST-3 is present in every WT tissue, in the tissues of every PC22 older than day 12, and in the fusion product between days 15 and 19. In P24 we could detect EST-3 only in protonemata exhibiting cytokinin induced budding. EST-3’ could only be detected in day 12 and, to a lesser extent, in day 15 WT protonemata with or without exogenously applied cytokinin. EST-4 is expressed in tissues of the wild type and the fusion product between days 15 and 19 with or without exogenously applied cytokinin. In P24 EST-4 is detectable only in protonemata exhibiting cytokinin-induced budding (day 12 + 21 days of cytokinin treatment).

MDH. The data of a number of experiments are presented in Fig. 7. Nine different MDH bands could be detected. MDH-3 to MDH-8 appear to be constitutive in all tissues investigated. Exclusively in 33-day-old WT with leafy gametophores MDH-3 is replaced by MDH-3’. 
Fig. 2. DAPI stained protonema of the fusion product PC22(+)P24.

Fig. 3. Example from one experiment detecting the EST isoenzyme pattern of 15-day-old *P. patens*, of two mutants and their somatic hybrid. A: WT; B: P24; C: P24(+)PC22; D: PC22(+) indicating treatment of 12-day-old protonemata for 3 days with 10^{-3} M 'Ade.
Fig. 4. Example from one experiment detecting the MDH isoenzyme pattern of 15-day-old *P. patens*, of two mutants and their somatic hybrid. A: WT; B: P24; C: P24(+)/PC22; D: PC22. ′ indicating treatment of 12-day-old protonemata for 3 days with $10^{-5}$ M 6-Ade.

Fig. 5. Example from one experiment detecting the PX isoenzyme pattern of 15-day-old *P. patens*, of two mutants and their somatic hybrid. A: WT; B: P24; C: P24(+)/PC22; D: PC22. ′ indicating treatment of 12-day-old protonemata for 3 days with $10^{-5}$ M 6-Ade.
which has a slightly higher mobility in the gel. MDH-1 is specific to the WT between days 12 and 19, while MDH-2 is only expressed in P24 at day 12 and in 33-day-old protonemata with or without cytokinin. PX. The data of a number of experiments are presented in Fig. 8. We could maximally detect five different peroxidase bands. Four of them (PX-1, PX-2, PX-4, PX-5) appear to be expressed constitutively in every tissue inves-

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Fig. 8. Complete PX isoenzyme patterns of *P. patens* (WT), two of its mutants, and their somatic hybrid (repeated experiments). * indicating treatment of protonemata with $10^{-6}$ M i'Aden from the 12th day on. d, (day(s).

tigated. PX-3, absent in the mutant P24, is expressed in the WT and in PC22 from day 15 on with or without exogeneously applied cytokinin. In the fusion product it is only detectable in 33-day-old protonemata.

### Discussion

Under our conditions the WT strain of *P. patens* and developmental mutants, derived from it, do not differ as much in the number of cytokinin-induced buds as they differ in the period of time the exogeneously applied growth regulator needs to induce these buds (compare Ref. 14). We therefore developed a highly reproducible and rather simple measure of cytokinin sensitivity in mosses, that is the time that $10^{-5}$ M i'Aden needs to induce buds in 12-day-old protonemata.

In this physiological test the wild type and

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Fig. 9. All detected differences in isoenzyme patterns of *P. patens* (WT), two of its mutants, and their somatic hybrid. * indicating treatment of protonemata with $10^{-6}$ M i'Aden from the 12th day on. d, day(s).
the fusion product reacted after three days, while PC22 does so after seven and P24 after 21 days. This time may be called the 'latent period' of the exogeneous cytokinin and appears to be positively correlated with the ability of the different genotypes to form buds without an applied growth regulator. For this reason the different lengths of the latent periods describe the different cytokinin sensitivities of the developmentally abnormal moss mutants and this may give an indication of the severity of the regulatory defects in these mutants.

The data obtained from this physiological test provide evidence for complementation in the fusion product. This encouraged us to study the physiological and genetic background of the WT, the two mutants and the fusant utilizing isoenzyme analysis. We separated proteins from various samples throughout the course of growth and differentiation of the four genotypes on native PAA-gels and stained them specifically for esterases, malate dehydrogenases and peroxidases.

The expression of several isoenzymes changed during growth and differentiation. These variations in isoenzyme patterns were reproducible and thus appeared to be specific for one genotype in the course of its differentiation. This might be due to mutations in the regulation of physiological processes. A comparison of the isoenzyme patterns of the four genotypes, however, reveals that there is no direct relation between these enzymes and budding, whether cytokinin induced or not (see Fig. 9).

Taking into account the difficulties that arise when interpreting physiological and isoenzyme data in terms of molecular events in an intraspecific hybridization, we would like to point out some probable events that could have taken place after the fusion of PC22 and P24. There are several possible fates for nuclear genes after somatic hybridization [15,16,27]: (i) The cells fuse, but the nuclei do not. If both nuclei are maintained, this would result in a dicaryotic plant or in a chimera. If one nucleus is segregated, the fusant would resemble one of the mutant traits. (ii) After protoplast fusion a nuclei fusion takes place. In the case of haploid moss protoplasts this would result in a diploid plant. After fusion of nuclei parts of the genome may be eliminated.

The WT-like cell and chloroplast morphology, the wild type-like cytokinin sensitivity and the ability of PC22(+)/P24 to produce leafy gametophores is strong evidence for complementation after electrofusion. Concerning the isoenzyme data, a further indication for complementation in the fusion product is that EST-4 is present in WT and PC22(+)/P24 between days 15 and 19 but that it can barely be detected in P24 (only at day 33 with cytokinin induced budding) and never in PC22. This finding makes the possible early elimination of one of the mutant genomes very unlikely.

Pelletier and Chupeau [27] suggested that most regenerated hybrids of higher plants might be chimeric. We could exclude this for the moss PC22(+)P24. Moss protonemata show tip growth and during the exclusively vegetative propagation of the fusion product, plants exhibiting one of the mutant traits with their altered morphology were never observed. To eliminate the possibility that PC22(+)/P24 was a dicaryotic plant, we stained protonemata of the fusion product with the DNA-specific fluorochrome DAPI. Every investigated cell possessed only one nucleus.

Taken together these observations indicate that a fusion of nuclei had taken place after electrofusion. This is considered to be the main event when hybridizing e.g. nearly related species [28,29] or after intraspecific hybridizations [30—32], which Hansen et al. [13] performed. Fahleson et al. [33] recently reported a high rate of nuclei fusion even when hybridizing rather distant species.

The isoenzyme pattern of the fusion product is not simply the addition of the patterns of both mutants, since MDH-2 is only expressed in P24. Furthermore, PX-3 could be detected in WT and PC22 from day 15 but in the fusion product it is expressed no earlier than day 33. This indicates that parts of the mutant genomes have been eliminated in PC22(+)/P24.

It is obvious that detailed molecular geneti-
cal analyses have to follow. As the data presented in this paper is rather encouraging, this type of experiments are underway.

Acknowledgements

This work was supported by Deutsche Forschungsgemeinschaft (Ab 10/8-2). F.Y. acknowledges a grant of the government of the Peoples Republic of China. The authors are grateful to Dr. D. Evans and Dr. W. Kasprik for reviewing the English text, to C. Adami for the photographic work and to A. Bräutigam for preparing the art work.

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