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PROTEIN SYNTHESIS DURING IN VITRO FLOWER BUD FORMATION IN TOBACCO

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SUMMARY

Protein synthesis was studied in relation to *in vitro* flower bud formation in tissue strips of tobacco. The rate of leucine incorporation increased from the 3rd day on up to the 7th day. Concomittantly an increment in protein content was observed. Protein synthesis was slow in control explants which did not produce buds. From the first day onwards the nature of the polypeptides synthesized *in vitro* differed from that of the protein already present in the original explants. However, in spite of the progression of development towards bud formation, there are only a few changes in the complement of the proteins being synthesized during this 6-day period.

1. INTRODUCTION

There is only scarce information on the synthesis of proteins specifically related to flowering (for review, see BERNIER et al. 1981). Studies in this field are hampered by the fact that in general flower buds are initiated at minute spots on grown-up plants and, as a consequence of the spacial arrangement, by the technical difficulties in directing radioisotopes specifically towards the bud initiating tissue.

These problems are largely overcome by the in vitro culture of strips cut from stem tissue of the inflorescence of tobacco (TRAN THANH VAN 1973a, b). On these explants which are open to nutrient and isotope uptake over their whole length, 20–30 flower buds develop rather synchronously within two weeks. In strips cut from the pedicels of the flowers, bud formation is the major developmental process taking place (VAN DEN ENDE et al. 1984b). The outlines of morphogenesis and the ways to manipulate bud initiation by hormone treatment have been well characterized (BARENDSE et al. 1985; VAN DEN ENDE et al. 1984b, c).

The present study deals with the quantitative and qualitative aspects of protein synthesis during flower bud formation in vitro. The main questions are focussed on the relation between protein synthesis and morphogenesis, especially in the early stages, and on the nature of the new proteins being formed.

2. MATERIALS AND METHODS

2.1. Plants and conditions of culturing

Plants of *Nicotiana tabacum* L. were grown from seed as described by VAN DEN

ENDE et al. (1984b, c). Strips of superficial tissue were cut from pedicels of flowers at anthesis (VAN DEN ENDE et al. 1984a) and cultured on Murashige-Skoog medium (MURASHIGE & SKOOG 1962) supplemented with growth regulators and glucose, 150 mmol l^{-1}. The standard medium contained 1 μmol l^{-1} of both benzyladenine (BAP) and α-naphthaleneacetic acid (NAA). A low-hormone medium containing a ten-fold lower concentration of both growth regulators on which no flower buds are initiated, has been used as the control.

2.2. Quantification of protein synthesis

For measuring the rate of protein synthesis samples of 5 explants were labeled for 6 h with 19.2 kBq ml^{-1} of [^{3}H]leucine added to the medium. The leucine concentration was 600 μmol l^{-1} unless stated otherwise. After incubation the tissues were immediately homogenized in 96 per cent ethanol and centrifuged. The pellet was washed once with 70 per cent ethanol and proteins were extracted with 1 mol l^{-1} NaOH at 37 °C overnight. Aliquots of the extracts were mixed with Aqualyte (Baker) and analyzed for radioactivity in a liquid scintillation analyzer (Philips PW-4540). Protein contents of the NaOH extracts were assayed with the Lowry method (LOWRY et al. 1951).

2.3. Qualitative analysis of proteins

For an analysis of the newly-synthesized proteins explants were labeled for 24 h with [^{3}H] or [^{14}C]leucine added to the medium. L-[4,5-^{3}H]leucine, 5.5 TBq $mmol^{-1}$ (Amersham) was provided at 0.37 MBq ml^{-1}. L-[U-^{14}C]leucine, 11 GBq $mmol^{-1}$, (Amersham) was given at 37 kBq ml^{-1}. At the end of the incubation period the explants were homogenized and extracted by the procedure of WEBER & OSBORN (1975). The proteins were separated by electrophoresis on an 11 per cent polyacrylamide gel. The distribution of counts was vizualized by fluorography (BONNER & LASKEY 1974). Occasionally proteins were stained by silver staining (ESCHENBRUCH & BÜRK 1982).

For a comparison of protein patterns two differently labeled extracts were mixed prior to electrophoresis. The ^{14}C labeled sample was prepared from strips incubated with [^{14}C]leucine on the first day. The ^{3}H-labeled proteins were obtained from strips incubated with [^{3}H]leucine on the 2nd, 4th or 6th day. Routinely, 11 kBq ^{3}H and 1.3 kBq ^{14}C were brought in one slot. After electrophoresis the gel lanes were sliced. Each slice was digested in a separate vial with 30 per cent H_2O_2 at 70 °C overnight. The digest was mixed with Aqualyte and assayed for radioactivity. The $^3H/^{14}C$ ratio was calculated for all slices of the lane. Proteins which are relatively over- or underrepresented in one of the original extracts will cause a sudden rise or dip in the $^3H/^{14}C$ ratio of one or two adjacent slices.

3. RESULTS

When strips of pedicel tissue are explanted on standard medium, the first flower buds appear 7 to 8 d after the onset of culture (VAN DEN ENDE et al. 1984b, c). Bud emergence is preceded by a number of morphological and physiological

events. The cells in the layers closest to the medium are actively dividing by the third day (VAN DEN ENDE et al. 1984c. At the same time the weight of the strips initiating buds begins to exceed that of the non bud forming controls (*fig. 1*). About one day earlier the protein content of the explants on the standard medium starts to rise (*fig. 2*) causing a temporary increase in the amount of protein per mg tissue. After 6 d in culture the initial ratio is restored. These results suggest that there is an intense synthetic activity in the interval between three and six days.

3.1. Kinetics of protein synthesis during early development

To elucidate the synthetic processes going on, we followed the incorporation of [^{3}H]leucine during the first 8 d of development. In such experiments the effect of isotope dilution by the endogenous unlabeled leucine pool must be minimized by enhancing the leucine concentration in the medium. A concentration of 600 µmol l^{-1} leads to saturation of leucine incorporation (*fig. 3*). This concentration was, therefore, used to study the kinetics of protein synthesis (*fig. 4*). The rate of leucine incorporation by bud-forming tissuses, compared with the control, shows already visible differences after three days which remain up to the 8th day in culture. At its maximum leucine incorporation in explants forming flower buds is about three times higher than in the control strips.

3.2. Qualitative differences in protein synthesis

The question arises whether the increase in leucine incorporation from the third day onwards leads to a change in the composition of the newly synthesized pro-

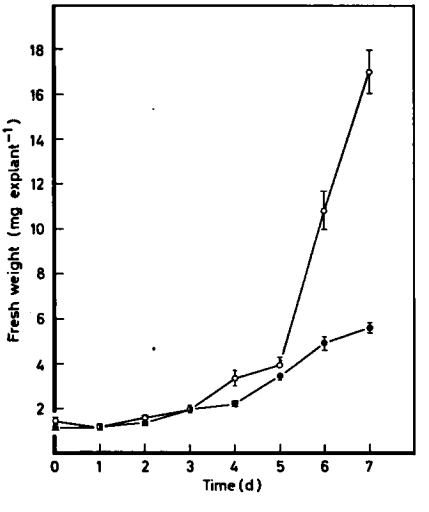

Fig. 1. Increase in fresh weight of explants during the first week of bud development. ○——○ tissues on standard medium; ●——● non bud forming controls. Vertical bars indicate standard errors of the means (n = 4).

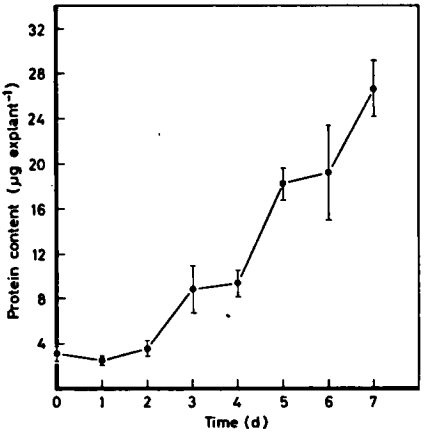

Fig. 2. Protein content of explants during the early stages of flower bud formation. Vertical bars indicate standard errors of the means (n = 3).

teins. Preferential synthesis of some proteins relative to other might accompany the initiation of new structures, i.e. the emerging buds. Tissue strips were labeled for 24 h on standard medium with [^{3}H]leucine on the first day and the subsequent days. Proteins were extracted, separated by gel electrophoresis, and vizualized by fluorography (*fig. 5a–d*). An unlabeled protein sample from strips was subjected to electrophoresis in the same way for comparison. The pattern revealed by silver staining shows the distribution of the pre-existing proteins (*fig. 5e*). Two conclusions can be drawn concerning the newly synthesized polypeptides. First, their distribution is completely different from the complement in the original explant. Furthermore, there are no major changes in the distribution of the protein synthesized during the first four days of development.

In search of minor differences in protein distribution which are not revealed by fluorography, we labeled the proteins with [^{3}H]leucine as before on three days starting at the second day of culture. Prior to electrophoresis the protein

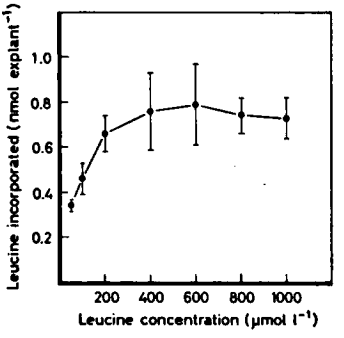

Fig. 3. Effect of the leucine concentration in the medium on the incorporation of [^{3}H] leucine by bud forming explants during the second day of development. Means and standard errors of the means were calculated on basis of 4 determinations.

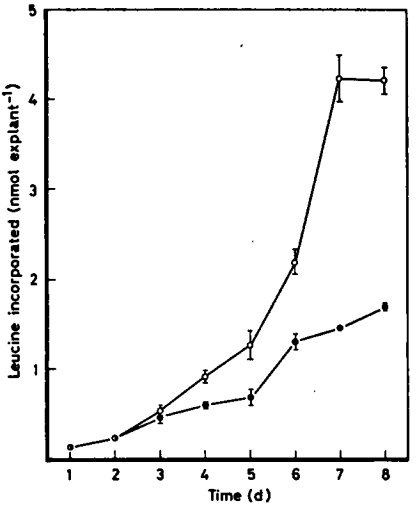

Fig. 4. Leucine incorporation in explants during the first period of flower bud development. Data points are means of 4 determinations. Other legends as in *fig. 1.*

extracts were mixed with proteins labeled at the first day with [^{14}C]leucine. The gels were sliced after electrophoresis and the ^{3}H/^{14}C ratio in each slice was determined as described in Material and Methods. Changes in the count ratio were consistently found at four positions in the gel (*table 1*). The relative abundance of a protein at one position increases after the first day whereas there is a decrease at three other positions. It is concluded that the synthesis of only a small number of proteins is preferentially stimulated or repressed during early bud development.

4. DISCUSSION

Flower bud initiation on pedicel explants takes place in about four days (VAN DEN ENDE et al. 1984b). By that time clumps of dividing cells, the division centres,

Table 1. Changes in the rate of synthesis of proteins at individual bands in the polyacrylamide gel. Deviation in the ^{3}H/^{14}C ratio of one or two adjacent slices (see Materials and Methods) are recorded relative to the background value which is calculated from the neighboring slices at both sides. (+) ratio increased; (−) ratio decreased. Deviation from background: (+, −) 10–15 per cent; (+ +, − −) 15–25 per cent; (− − −) more than 25 per cent.

Position (R_f)	Time (d)		
	2nd	4th	6th
0.33	− −	− − −	− −
0.60	−	−	− −
0.78	+ +	+ +	+ +
0.83	−	−	−

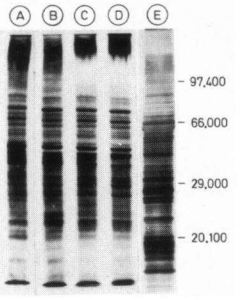

Fig. 5. Distribution on polyacrylamide gels of proteins synthesized during the (a) first, (b) second, (c) third, (d) fourth day of development. The proteins pre-existing in the strips are silver-stained and shown in lane (e). Approximate molecular weights are given at the right hand side of the figure. Marker: Phosphorylase B (97,400 D), bovine serum albumin (66,000 D), carbonic anhydrase (29,000 D), and trypsin inhibitor (20,100 D).

are formed from which the flower buds eventually arise. A number of physiological changes accompany this development. The rate of protein synthesis and protein start to rise after three days (*figs. 2, 3*). One day later the tissue fresh weight follows (*fig. 1*). Protein content as a percentage of tissue weight is temporarily enhanced. This reflects the presence of a large number of small rapidly dividing cells with small vacuoles. The increase in fresh weight and leucine incorporation on the low-hormone medium which does not support bud formation indicates that not all physiological changes in explants on standard medium are exclusively related to bud development.

The distribution on polyacrylamide gels of the polypeptides synthesized during the first period in culture differs profoundly from the protein present in the explant at the time of incubation (*fig. 5*). At first sight this seems suprising since the long labeling period (24 h) favours the accumulation of counts in the stabile proteins. It should be realized, however, that the conditions in tissue culture are very dissimilar from those in the intact plant. There is, in contrast, no indication for major changes in protein synthesis during the first 6 days of bud development (*fig. 5, table 1*). This means that the increase in the rate of protein synthesis in this period relates to all but a few major proteins in a proportional fashion. That only a few proteins behave differently during the rapid and rather synchronous development of a large number of buds underlines that only a few major proteins are involved in the start of this process. Analogous results have been reported for a variety of developmental processes (BATES & CLELAND 1980, GUERRI et al. 1982, MEYER et al. 1984).

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