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Protein Synthesis Associated with Quiescence and Senescence in Auxin-Starved Pear Cells[1]

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ABSTRACT

Pear fruit cells (*Pyrus communis* L. cv Passe Crassane) stopped dividing when subcultured in a bioreactor under auxin starvation in the presence of 0.37 molar mannitol. The cessation of cell division was preceded by the accumulation of a specific basic polypeptide of 24 kilodalton. Readdition of 2.3 micromolar 2,4-dichlorophenoxyacetic acid (2,4-D) neither caused a resumption of cell division nor depressed the accumulation of this polypeptide. Under complete auxin starvation, cells began to die at day 18. *In vivo* radioactive labeling of proteins followed by two-dimensional electrophoresis showed that during auxin starvation the synthesis of some polypeptides including the 24 kilodalton one (referred to as homeostasis-related proteins, HRPs) was decreased while the synthesis of some others (referred as senescence-related proteins, SRPs) was increased. Readdition of 2.3 micromolar 2,4-D postponed the onset of cell death by 10 to 15 days while supplementation with 7.6 micromolar abscisic acid advanced cell death by 8 days. Two-dimensional analysis of protein synthesis indicated that both hormones interact on the synthesis of these two groups of polypeptides. The levels of most HRPs were maintained or increased in the presence of auxin, while the levels of the SRPs were decreased by auxin and increased by abscisic acid. Short and long-term effects of 2,4-D and abscisic acid on the synthesis of specific polypeptides were observed, allowing a discrimination between the direct and indirect effect of both hormones on the development of cell senescence.

Senescence has been associated with the quantitative loss of (or the loss of sensitivity to) auxins and cytokinins (20). Indeed, auxin or cytokinin applications to plant tissues delay senescence (20, 25, 27). Furthermore, auxin deprivation of plant cells in culture results in the development of physiological and ultrastructural changes similar to those occurring in senescing tissues (1–3).

Characteristic changes in the rates of synthesis of individual proteins have been observed upon auxin deprivation of suspension cultured cells or protoplasts (5, 12, 13, 15). The physiological role of proteins induced during auxin deprivation has recently received more attention. In tobacco cells, auxin or cytokinin starvation triggers a dramatic increase of β-glucanase m-RNA level after a few days (16). Moreover, it was shown that all the polypeptides whose synthesis is decreased by auxin in tobacco cells are located in the vacuole (15). So far, their involvement in the development of cell senescence and death as well as the modulation of their synthesis by senescence retarding or senescence accelerating hormones has not been studied.

A system of quiescent cells in culture was developed previously (6) consisting of 2,4-D-deprived quiescent pear cells cultured in a closed continuous bioreactor. It has been used to study the role of auxins and other hormones in the senescence process (1, 3).

This paper deals with the development of cell senescence upon auxin starvation and its delay or acceleration by low levels of 2,4-D or ABA. It reports on the synthesis of specific proteins during the transition from quiescence to senescence. Attempt is made to discriminate between polypeptides involved in the maintenance of cellular integrity (HRPs)[2] and polypeptides more directly associated with the senescence process (SRPs). Finally, the role of auxin and ABA in the synthesis of these proteins is investigated.

MATERIALS AND METHODS

Maintenance of Cell Suspensions and Culture Conditions. A cell strain of Passe-Crassane pear (Pyrus communis L.) established in 1981 from young fruits was used. The cell suspensions were grown as previously described (6, 19) in a standard medium supplemented with 4.5 μM 2,4-D.

After 9 d of culture in the standard medium minus 2,4-D, cells were transferred into a closed-continuous bioreactor (1) containing 2 L of 'aging medium' (one-fourth concentration of the mineral and organic nutrients present in the standard medium, plus 0.37 M mannitol and 0.03 M sucrose). The final cell density was always around 10^6 cells ml^{-1}. The effects of 2,4-D (or ABA) were examined after supplementing at d 7 both the bioreactor and reservoir media with 2.3 μM 2,4-D or 7.6 μM ABA.

Radioactive Labeling of Cells. A 2.5 ml aliquot of cell suspensions containing around 10^6 cells $\cdot ml^{-1}$, was aseptically transferred into a 25 ml flask. Labeling was performed during various periods of time (see below) at 25°C under gentle shaking in the presence of 740 kBq [^{35}S]-methionine (41.1 TBq $\cdot$ $mmol^{-1}$, CEA, France) or in the presence of 555 kBq [^{14}C]leucine (11.1 GBq $\cdot$ $mmol^{-1}$, CEA, France). Rapid changes induced by hormones were estimated according two procedures: (a) 20 h labeling: hormones and radioactive precursors were added simultaneously and labeled proteins were extracted after 20 h; (b) 4 h labeling: after supplementation with hormones of several flasks at time 0, radioactive precursors were added at 4 h intervals and labeled proteins were extracted after 4 h of incubation. All operations were done under sterile conditions.

Extraction of Proteins for Electrophoresis. The procedure was adapted from Meyer *et al.* (15). After labeling, cells were filtered

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[2] Abbreviations: HRPs, homeostasis-related proteins; SRPs, senescence-related proteins.

through a Buchner type funnel, rinsed twice with 15 to 20 ml of 0.9% (w/v) NaCl, frozen in liquid N_2, and stored at −20°C before freeze-drying. Ten mg of freeze-dried cells were then dry-ground at low temperature in a homogenizer equipped with Eppendorf microtubes (1.5 ml). After precipitation of proteins for 2 h in 1.0 ml of 15% (w/v) TCA, the pellet was recovered by centrifugation (5000*g*), rinsed once with 1 ml of 15% TCA, and five times with 1 ml of 0.1 M NH_4HCO_3 in methanol. It was then dried under vacuum at 4°C.

For two-dimensional electrophoresis, the protein pellet was redissolved in a solution of 9.5 M urea containing 5% (v/v) 2-mercaptoethanol and 2% (v/v) NP 40. After centrifugation (30,000*g*), the supernatant was supplemented with 2% (v/v) ampholines (LKB pH 3.5–10) and run immediately or stored at −20°C until electrophoresis.

For one-dimensional SDS-PAGE, the protein pellet was dissolved in 62.5 mM Tris-HCl (pH 6.8) containing 10% (v/v) glycerol, 5% 2-mercaptoethanol, and 2.3% (w/v) SDS. After heating 3 min at 100°C, the soluble material was recovered by centrifugation (30,000*g*) and used for SDS-PAGE electrophoresis.

In some experiments mentioned in the text, the recovery of "soluble" proteins refers to extracting dry-ground cells with 10 mM Tris-HCl (pH 7.4), containing 5 mM $MgCl_2$ and removing insoluble material by centrifugation (30,000*g*—10 mn). For SDS-PAGE, the supernatant was adjusted to 7.5% 2-mercaptoethanol, 10% glycerol, 62.5 mM Tris-HCl (pH 6.8) and 2.3% SDS. After boiling 3 min, the extract was ready to use.

Electrophoresis. SDS-PAGE was carried out according to Laemmli (9) using a 4% stacking and a 12.5% separating gel. The two-dimensional system described by O'Farrell *et al.* (18) included a NEPHGE gel in the first dimension followed by SDS-PAGE as modified by Meyer and Chartier (13). Visualization of labeled proteins was achieved by fluorography. Kodak X-OMAT-S films were exposed for appropriate times (4 d–8 weeks) at −70°C. The following mol wt markers were used: lysozyme (14.3 kD), trypsinogen (24.0 kD), ovalbumin (45.0 kD), BSA (66.0 kD). The acidic and basic ends of the two-dimensional gels (respectively located on the right and left side of the figures) represent the 3.5 and 10 pH ends of the first dimension.

RESULTS

Changes in the Number of Live Cells during Subcultures. Cells were subcultured every 7 d in the presence of 2,4-D or occasionally transferred in the absence of 2,4-D. In the presence of 4.5 μM 2,4-D in the standard medium (step 1), pear cells undergo a classical growth cycle with a doubling time of approximately 72 h and a final cell density of about 5×10^6 cells ml^{-1} (Fig. 1A). Upon transferring to the standard medium lacking 2,4-D (step 2), the number of cell divisions was reduced, the stationary phase was soon initiated with a final density of only 2×10^6 cells ml^{-1}, and cells began to die after 9 d (Fig. 1B). When 7-d-old cells from step 2 were transferred into a continuous bioreactor fed with an aging medium also lacking 2,4-D but containing mannitol (step 3), no division occurred and cells remained alive for at least 15 d in the total absence of 2,4-D (Fig. 1C). When the medium was supplemented with 2.3 μM 2,4-D at d 7, cells remained alive for at least 30 d. In contrast, 7.6 μM ABA advanced the timing of cell death by 8 d in the presence or absence of 2,4-D.

Accumulation of Characteristic Proteins in Quiescent Cells. The protein pattern of pear cells in culture was followed at selected periods under various physiological conditions in batch or continuous cultures. Polypeptides were extracted in the presence of the SDS detergent and separated by SDS-PAGE electrophoresis. The most significant difference between the various patterns presented in Figure 2 was the accumulation of a polypeptide of 24 kD, in cells entering quiescence. This polypeptide, began to accumulate prior to the onset of the stationary phase and reached a maximum in quiescent cells: 12 d in the presence of 2,4-D (Fig. 2, lane 4), 5 d in absence of 2,4-D (Fig. 2, lane 7),

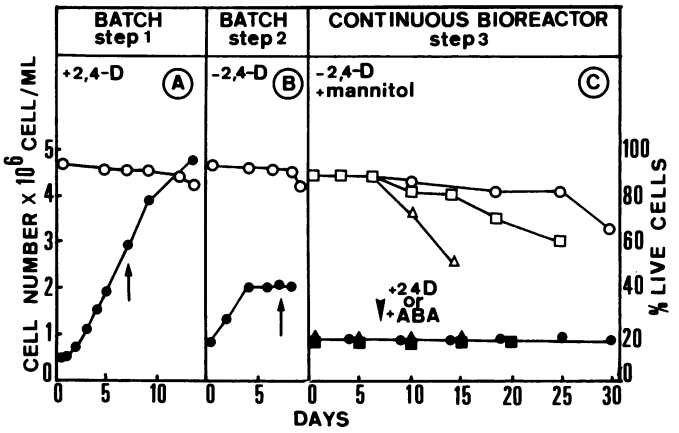

FIG. 1. Changes in total cell number and percent of live cells during successive subcultures in batch in the presence (A) or absence (B) of 2,4-D and in aging medium in the bioreactor (C). Quiescent cells in the bioreactor were either cultured in the total absence of 2,4-D (□, ■) or supplemented at d 7 with 2.3 μM 2,4-D (○, ●) or 7.6 μM ABA (△, ▲). Open symbols, % live cells; closed symbols, total cell number. Long arrows indicate the dates of transfer.

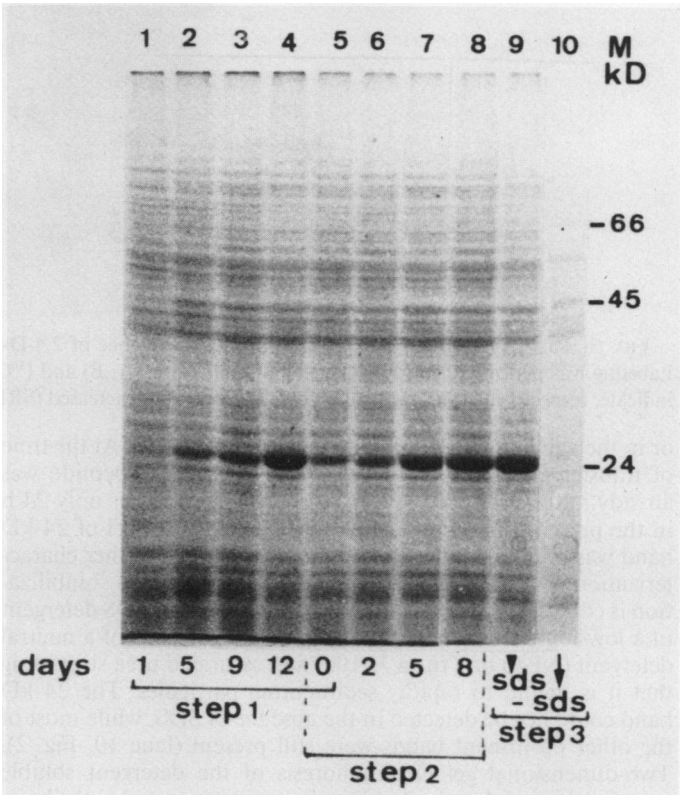

FIG. 2. SDS-PAGE electrophoresis of polypeptides from dividing or quiescent pear cells. Lanes 1 to 4, cells sampled at d 1, 5, 9, and 12 during a growth cycle in batch in the presence of 4.5 μM 2,4-D. Lanes 5 to 8, cells sampled at d 0, 2, 5, and 8 during a first transfer in batch in the absence of 2,4-D. Lanes 9 and 10, quiescent cells sampled at d 5 in the bioreactor in the total absence of 2,4-D. Polypeptides of lanes 1 to 9 were extracted in the presence of SDS and polypeptides of lane 10 in the absence of SDS. Polypeptides were stained with Coomassie blue.

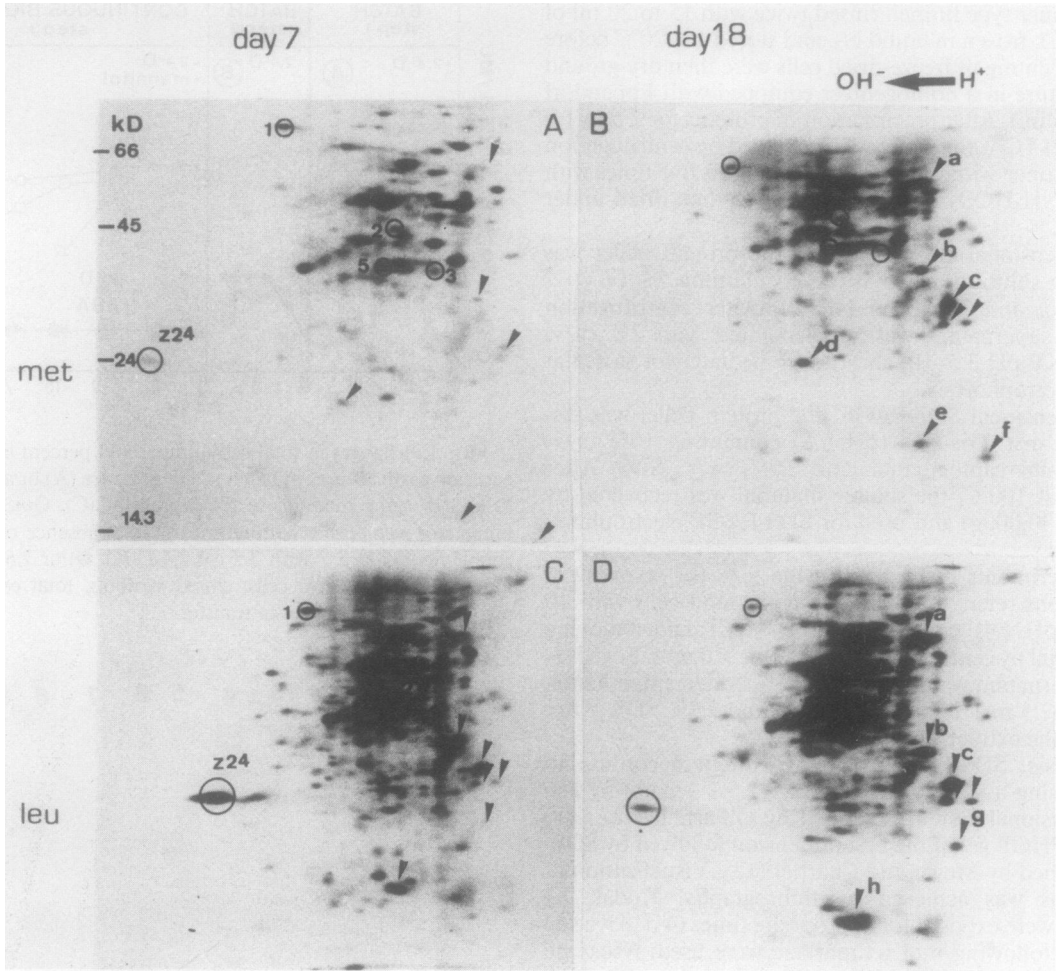

FIG. 3. Pattern of labeled polypeptides during senescence of 2,4-D-starved pear cells cultured in the bioreactor in the total absence of 2,4-D. Labeling was performed for 20 h using [^{35}S]methionine (A, B) and [^{14}C]leucine (C, D) as precursors. A, C = d 7; B, D = d 18. Arrows or circles indicate, respectively, the polypeptides whose synthesis is increased (SRPs) or decreased (HRPs) during the senescence process.

or in the aging medium minus 2,4-D (Fig. 2, lane 9). At the time of transfer every 7 d the level of the 24 kD polypeptide was already rather high (d 0 of steps 1 or 2, lane 5). After only 24 h in the presence of auxin a rapid decrease in the level of 24 kD band was observed (lane 1 compared to lane 5). Further characterization of the 24 kD polypeptide indicated that its solubilization is completely dependent upon the presence of SDS detergent in a low-ionic strength buffer or upon the presence of a neutral detergent (NP40 or Triton X-100) and saturated urea suggesting that it is bound to rapidly sedimenting particules. The 24 kD band could not be detected in the absence of SDS, while most of the other prominent bands were still present (lane 10, Fig. 2). Two-dimensional gel electrophoresis of the detergent soluble proteins showed that the 24 kD polypeptide migrated in the basic area of the gel. It could be detected as a unique and major polypeptide upon staining with Coomassie blue (not shown) or radioactive labeling (Fig. 3). As in the SDS-PAGE gel (Fig. 2), it was the most prominent polypeptide and it was far more intensely labeled in the presence of [^{14}C]leucine than [^{35}S]methionine (Fig. 3, A and C), suggesting that it has a low methionine content.

Changes in Protein Synthesis during Cell Senescence. Cells at different stages of senescence (after 7 and 18 d in bioreactor) were labeled in their original medium for 20 h with [^{35}S]methionine (Fig. 3, A and B) or [^{14}C]leucine (Fig. 3, C and D). Two-dimensional electrophoresis indicated a decrease, during the development of senescence, of several polypeptides indexed as 1, 2, 3, and 5 in Figure 3. These polypeptides also included the 24 kD band. In contrast, several other polypeptides indexed as a, b, c, d, e, f, g, and h were labeled at an increasing rate as cell mortality increased (Fig. 3).

The life span of these polypeptides was estimated by comparing the patterns of polypeptides detected after a short time labeling of 2 h (Fig. 4, A and B) or a long time labeling of 20 h (Fig. 4, A′ and B′). The intensity of labeling was very similar indicating that most of these polypeptides had a slow turnover. However, some spots, including c, e, and j, were comparatively less intensely labeled after 20 h and could therefore be considered as having a rapid turnover. These three polypeptides were synthetized at a higher rate in senescing cells (Fig. 4B as compared to Fig. 4A). One polypeptide, m, appeared to decrease in turnover during senescence (Fig. 4).

Effect of 2,4-D on Protein Synthesis during Senescence. Quiescent cells cultured in the bioreactor were supplemented with 2.3 μM 2,4-D in the bioreactor at d 7 (Fig. 1B) and their polypeptide patterns were compared with those of control cells cultured in the total absence of 2,4-D. During the first 20 h of treatment (procedure 'a' in "Materials and methods") the synthesis of eight [^{35}S]methionine labeled polypeptides (indexed as 2–7) were (re-)induced (Fig. 5, A, A′, B, B′). These changes were already observable (data not shown) 4 h after addition of 2,4-D (procedure 'b' in "Materials and Methods"). Labeling of proteins with [^{14}C]leucine indicated that synthesis of the 24 kD polypeptide was slightly decreased during the same period (not shown). The

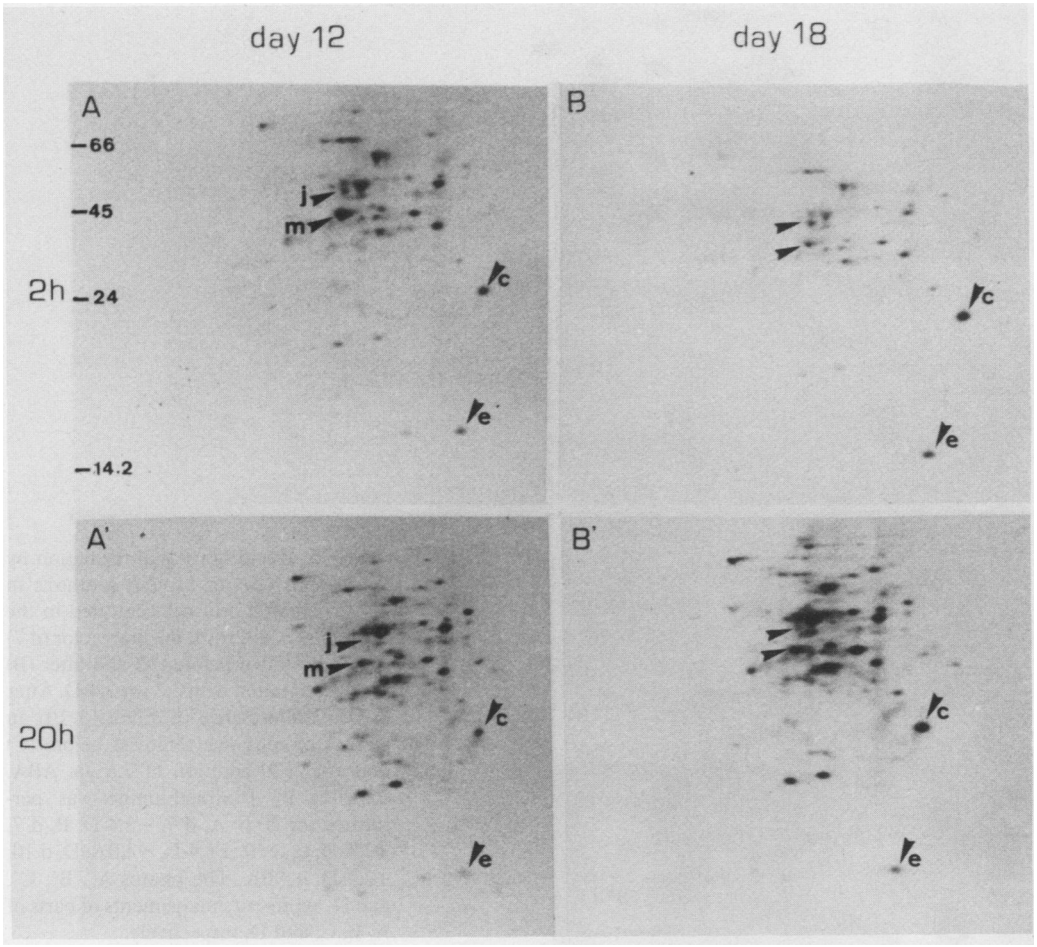

FIG. 4. Comparison of short-term and long-term labeling of polypeptides during senescence of 2,4-D-starved pear cells. Labeling was carried out for 2 h (A, B) or 20 h (A′, B′) in the presence of [^{35}S]methionine. The two-dimensional electrophoretograms refer to cells sampled at d 12 (A, A′) and d 18 (B, B′) in the bioreactor. The meaning of the symbols is the same as in Figure 3.

other polypeptides previously described (Fig. 3) showed no change in labeling during the same period.

A few days after the addition of 2,4-D (d 13), while the percentage of dead cells was still similar in both auxin treated and control cells, a reduction or an inhibition of the synthesis of polypeptides a, b, c, h, j, and l could be observed (Fig. 6). Thus, auxin delayed cell death and limited the associated increase in the synthesis of these particular proteins. In addition, 2,4-D maintained or stimulated the synthesis of some other polypeptides, *e.g.* the band at 24 kD, 2, 3, 4, 5, 6, 6′, and 6″ whose synthesis was depressed or very low during senescence.

Effect of ABA on Protein Synthesis during Senescence. Treatment with 7.6 μM ABA resulted in an increased synthesis of polypeptides b, c, d, e (not shown), and a reduction in the life span of the cells (Fig. 1).

When cells were first cultured in the presence of 2.3 μM 2,4-D and supplemented 3 d later with ABA, polypeptides b and c were more intensely labeled after 20 h while labeling of polypeptides 4, 5, 6, 6′, 6″, and 7 was decreased (Fig. 5, C, C′, D, D′). The 4 h labeling procedure led to the same conclusions (data not shown).

A summary of the major changes affecting protein synthesis is presented in Table I. Polypeptides whose synthesis increased during senescence (SRPs) are identified by letters, while those whose synthesis was reduced (HRPs) are identified by numbers. The first group preferentially included the most acidic proteins with a mol wt ranging from 14,000 to 80,000. Some of them (g and h) were very weakly labeled in the presence of [^{35}S]methionine. Most of the HRPs were neutral polypeptides of 24 to 45 kD and were more intensely labeled in the presence of [^{35}S]-methionine. Another distinction could be made according to the time elapsed from the addition of 2,3 μM 2,4-D or 7.6 μM ABA to the observable changes in protein synthesis. The reduction in synthesis of most SRPs was not detectable until a few days after auxin application, while the stimulation in synthesis of most HRPs was rapid (a few hours). The effect of ABA on synthesis of several SRPs (b, c, and d) and HRPs (4, 5, 6, 7) could be observed after a few hours.

DISCUSSION

The results reported here are in line with a few previous observations that qualitative and quantitative changes occur in the spectrum of proteins during the senescence of various plant systems including pea epicotyls (21, 22), soybean cotyledons (23), or volvox somatic cells (8). Determination of the involvement of specific proteins in senescence implies the possibility to modulate the rate of senescence and eventually to reverse it. In the soybean cotyledon system, rejuvenation by epicotyl removal is possible and some polypeptides were shown to increase with age and decline upon rejuvenation. However, there were no data on the regulatory role of plant hormones in the synthesis of proteins associated with an acceleration or delay of cell death.

The work reported here used a system of cells cultured *in vitro* which allowed manipulation of the level of hormones to modulate cell division and cell senescence, and therefore, to distinguish between polypeptides associated with the cessation of cell division, the promotion of senescence, or the maintenance of cellular functions.

Protein Synthesis Related to the Arrest of Cell Division. Concomitantly with the cessation of cell division, the 24 kD polypeptide accumulated in large amounts prior to onset of the stationary phase in the standard medium and in quiescent cells

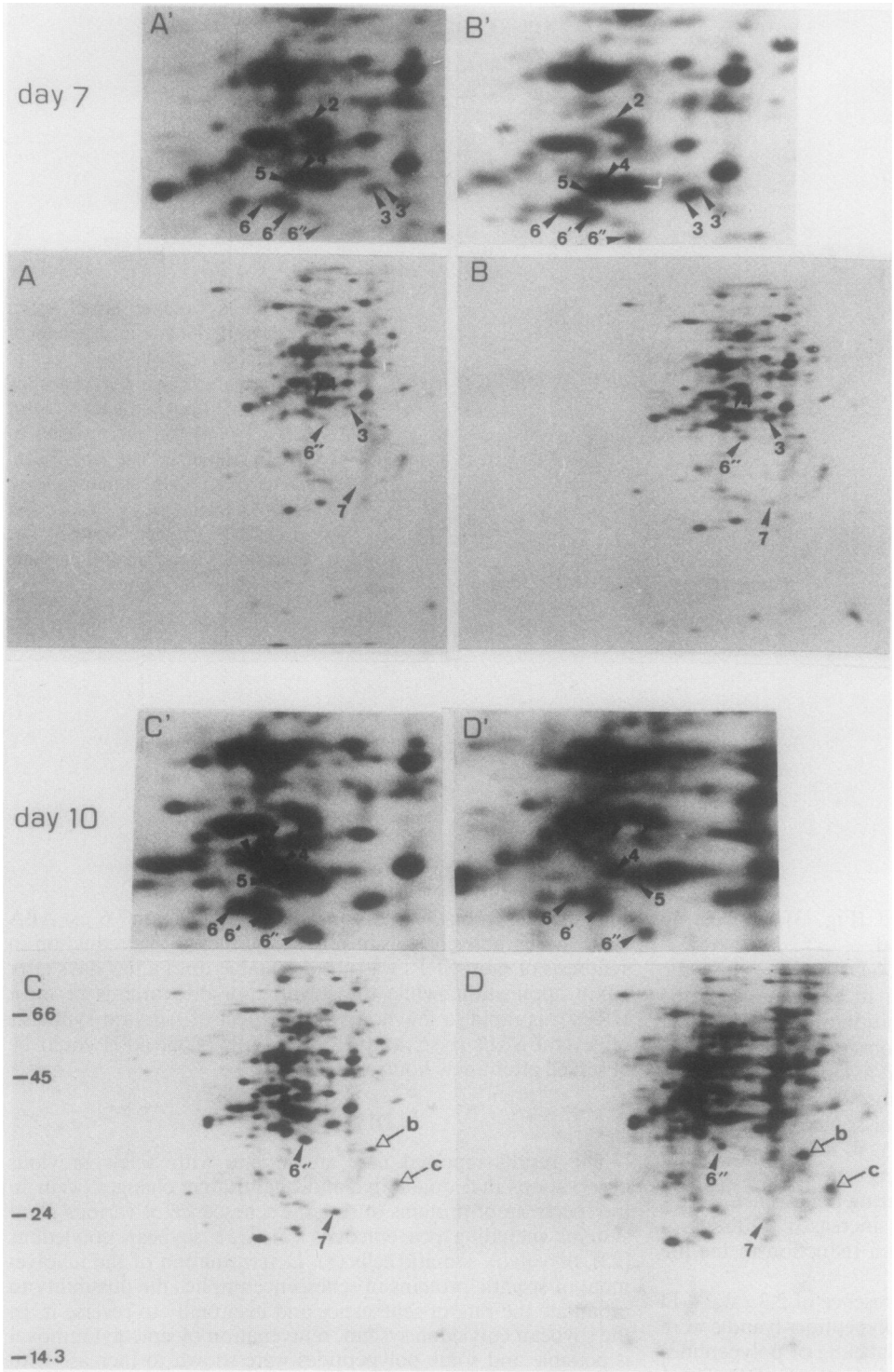

FIG. 5. Rapid hormonal regulation by 2,4-D and ABA of protein synthesis in 2,4-D deprived pear cells cultured in the bioreactor. Cells from the bioreactor (d 7) were withdrawn before (A) and after (B) supplementation with 2.3 μM 2,4-D. After 3 d in the presence of auxin (d 10) an aliquot of cells was removed before (C) and after (D) addition of 7.6 μM ABA. Labeling by [^{35}S]methionine was performed for 20 h. A, d 7, −2,4-D; B, d 7, +2,4-D; C, d 10, +2,4-D, −ABA; D, d 10, +2,4-D, +ABA. The photos A′, B′, C′, and D′ represent enlargements of parts of A, B, C, and D, respectively.

cultured in the bioreactor in the presence of mannitol. Readdition of 2,4-D to quiescent cells in the bioreactor in the presence of mannitol did not induce cell division and had no major effects on the 24 kD polypeptide accumulation or synthesis. In contrast, after transfer in a standard medium provided with 2,4-D, a resumption of cell division was observed associated with a sharp decrease in the level of the 24 kD band. It can, therefore, be concluded that the increased synthesis is more related to the cessation of cell division than to auxin deprivation per se. This 24 kD polypeptide revealed some analogies with a 26 kD protein detected in tobacco cells entering the stationary phase (10). This polypeptide was also shown to accumulate when the growth rate of the cells was reduced either by high concentration of NaCl or by ABA (10). The accumulation of major polypeptides has also been reported in auxin deprived soybean cells (12), tobacco callus (16), or tobacco cell suspensions (14, 15).

Senescence and Homeostasis-Related Proteins. The results reported here indicate that cell senescence is associated with an increased synthesis of several polypeptides (a, b, c, d, e, f, g, and h). Even though the physiological function of these polypeptides remains to be elucidated, they seem very likely involved in senescence. The increased synthesis of some polypeptides or enzymes has also been observed during aging of soybean cotyledons, pea epicotyls, and during leaf senescence (11, 21, 23, 25).

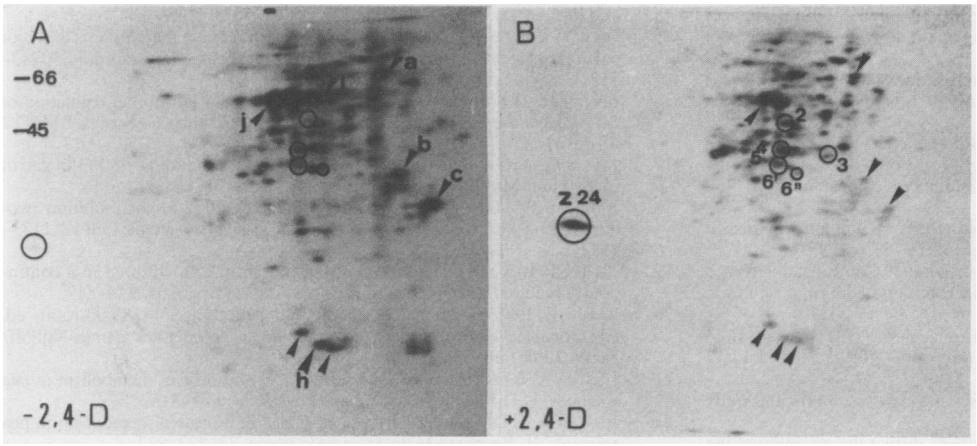

FIG. 6. Comparison of two-dimensional protein patterns of 13-d-old quiescent cells cultured in the absence of 2,4-D (A) or after readdition of 2.3 μM 2,4-D at d 7 (B). Radioactive labeling was performed for 20 h using [^{14}C]leucine. The meaning of the symbols is the same as in Figure 3.

Table I. *Major Changes in Protein Synthesis in Pear Cells during Senescence and in Response to Added 2,4-D or ABA*

	Polypeptides	kD	−2,4-D (senescence)	2.3 μM 2,4-D		7.6 μM ABA		Predominant Amino Acid labeling	Position in Gels	Turnover Rate[a]
				20 h	6 d	20 h	6 d			
SRPs	a	80	+[b]	0	−	0	+		Acidic	+
	b	36	+	0	−	+	+		Acidic	+
	c	29	+	0	−	+	+	leu	Acidic	+++
	d	22	+			+	+	met	Neutral	+
	e	15	+	0	−			leu	Acidic	+++
	f	14.5	+	0	−			met	Acidic	+
	g	22	+	0	−			leu	Acidic	
	h	15	+	0	−			leu	Neutral	
	j	48	+	0	−			leu	Neutral	+++
	l	50	0	0	−				Neutral	
	m	43	+	0	−			leu	Neutral	++/+
HRPs	24 kD	24	−	−	+	0	−	leu	Basic	+
	1	80	−	0	0				Basic	
	2	45	−	+	+	0	0	met	Neutral	+
	3[c]	36	−	+	+	0	0	met	Acidic	+
	4	38	0	+	+	−	−	met	Neutral	+
	5	38	−	+	+	−	−	met	Neutral	+
	6[c]	36–38	0	+	+	−	−		Neutral	+
	7	24	0	+	+	−	−	met	Neutral	+++

[a] Estimated visually by comparing patterns of polypeptides after 2 h and 20 h labeling. [b] +, increased; 0, no change; −, reduction. [c] Family of polypeptides.

Earlier observation had shown that the senescence of auxin-deprived pear cells was associated with the appearance of multivesicular bodies and membrane vesicles connected with the plasmalemma (2). These microbodies could be involved in the secretion of protein from the cytoplasm towards the cell wall. A similar phenomenom was described in tobacco leaves infected with tobacco mosaic virus (7), in association with the synthesis of pathogenesis related proteins (7). However, no convincing analogies seem to exist between SRPs and pathogenesis related proteins despite some common traits like acidity and low labeling with [^{35}S]methionine (26).

Senescence of pear cells *in vitro* was also associated with a reduced synthesis of several polypeptide species: 24 kD, 1, 2, and 3. A decrease in synthesis of several proteins or enzymes has also been reported during senescence of other plant systems (8, 20, 21, 23).

Our results indicate that ABA and 2,4-D interact on the synthesis of some specific proteins, associated with cell viability (HRPs) or cell senescence (SRPs) in cultured pear cells. However, reduction in synthesis of all the SRPs by 2,4-D is a long-term response, suggesting an indirect action of 2,4-D on cell senescence. The long-term regulation of synthesis of some polypeptides by auxins had already been found in other systems (4, 24) and the indirect role of auxins and cytokinins on plant senescence was previously suggested in leaf senescence (25). In contrast, the effects of 2,4-D and ABA on the synthesis of some HRPs is a short-term response. The hormonal regulation of synthesis of specific polypeptides and expression of specific genes is now clearly established (17, 24). The short-term modulation by ABA and 2,4-D of these polypeptides suggests a direct regulatory function for some of them.

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