Metabolism of proteins in developing leaves of oats (*Avena sativa* L.)

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Proefschrift

ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr. H. C. van der Plas,
in het openbaar te verdedigen
op donderdag 12 oktober 1989
des namiddags te vier uur in de aula
van de Landbouwuniversiteit te Wageningen.

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Stellingen:

1. In tegenstelling tot wat er meestal in de literatuur vermeld wordt, is bladveroudering niet geassocieerd met synthese van specifieke nieuwe eiwitten maar met het voortduren van de synthese van een set hoog- moleculaire eiwitten met een hoge turnoversnelheid.

(Dit proefschrift)

2. Resultaten uit studies met afgesneden bladeren (organen) mogen niet worden geextrapoleerd naar bladeren (organen) aan de plant.

(Dit proefschrift).

3. Het wordt helaas te weinig onderkend dat resultaten verkregen met *in vitro* translatie van mRNA's pas zinvolle informatie opleveren over een ontwikkelingsproces, als ze worden vergeleken met die van eiwitsynthese *in vivo*.

(Dit proefschrift).

4. Het is onjuist om het rijpen van vruchten als veroudering te beschrijven.

5. Bij onderzoek van de stikstoffixatie door *Rhizobium* wordt ten onrechte de invloed van de fysiologische toestand van de gastheerplant sterk verwaarloosd.

6. Het is niet aangetoond dat microbiële sideroforen een rol spelen bij de ijzeropname van hogere planten.

7. De resultaten van Shimazaki *et al.*, (1989) zijn niet voldoende overtuigend om de hypothese dat ribulosebisfosfaatcarboxylase/oxygenase afwezig is in sluitcellen van huidmondjes van *Vicia faba* L. te verwerpen.

Shimazaki K, J Terada, K Tanaka, and N Kondo (1989) Calvin-Benson cycle enzymes in guard-cell protoplasts from *Vicia faba* L. Implications for the greater utilization of phosphoglycerate/dihydroxyacetone phosphate shuttle between chloroplast and cytosol. Plant Physiol 90; 1057-1064

Tarczynski MC, WH Outlaw Jr, N Arnold, V Neuhoff and R Hampp (1989) Electrophoretic assay for ribulose 1,5 - bisphosphate carboxylase/oxigenase in guard cells and other leaf cells of *Vicia faba* L. Plant Physiol 89; 1088-1093

8. Ten onrechte beweert Mandelbrot dat de vaak sterke overeenkomst van fractale geometrische figuren met vormen en grafische representaties van processen in de natuur een toepassing van deze wiskunde in de natuurwetenschappen mogelijk maakt.

Mandelbrot BB (1983) The fractal geometry of nature. Freemond and Company, NY.

9. Het MS-DOS compatibel zijn van een computer is meer een min dan een plus punt.

10. Het wordt vaak vergeten dat natuurbeheer meer een noodzakelijk kwaad is dan een noodzaak op zichzelf.

11. Fanatiek feminisme leidt tot vrouwen discriminatie door vrouwen.

Voller D (1988) Madonna the biography, Omnibus Press.

Aan mijn Ouders

Voorwoord

Het tot stand komen van een proefschrift is niet alleen de verdienste van de promovendus. Een groot aantal mensen hebben er mede toe bijgedragen dat het proefschrift in deze vorm kon worden voltooid. In dit voorwoord wil ik deze mensen hartelijk bedanken:

Professor Bruinsma, mijn promotor, wil ik bedanken voor de vele waardevolle opmerkingen tijdens de werkbesprekingen en vooral de steun die ik van hem heb ondervonden tijdens de laatste periode van het schrijven van dit proefschrift.

Mijn co-promotor, Dr. ir. L.C. van Loon, geestelijk vader van het project en dagelijks begeleider wist mij steeds weer te motiveren als ik door de vele eiwitspots in paniek begon te raken. Ik heb hopelijk veel geleerd van zijn analytische manier van denken en accurate wijze van formuleren.

De studenten Edwin Bakx, Mariken Rebers en Ad Jespers, annalisten Yvonne Gerritsen en Cor Meurs en gastmedewerkster Susan Tophof hebben allen veel 2D-PAGE gels gemaakt en een belangrijke bijdragen geleverd. Allen droegen zij ook bij tot de plezierige werksfeer in onze groep.

Met Henry van der Valk, mijn kamergenoot van het eerste uur in een parallelproject, heb ik altijd met plezier samengewerkt. Na soms verhitte discussies werden hypothesen bedacht en verworpen. Verder heeft Henry mij in de eerste maanden wegwijs gemaakt in het reilen en zeilen van de vakgroep.

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Naast de mensen die direct met het proefschrift te maken hebben gehad hebben veel mensen van de vakgroep door hun belangstelling en kameraadschap een belangrijk deel gehad aan de sfeer die gedurende mijn onderzoek heerste. Vooral de volgende mensen wil ik bedanken: Frank Dumoulin, een goede vriend en toeverlaat, Monique Wijdeveld voor de altijd vrolijke uitstraling, Erik Knegt, mede computer enthousiast, met wie het op de verschillende computerbeurzen goed toeven was (en nog steeds is) en die altijd tijd had om over mijn experimenten te praten.

Ook de andere medewerkers van de vakgroep die ik niet met name heb genoemd wil ik bedanken. Zonder jullie was dit boekje echt niet afgekomen.

Contents

List of abbreviations

2D-PAGE	two-dimensional polyacrylamide gel electrophoresis
BA	N^6-benzyladenine
BSA	bovine serum albumine
EDTA	ethylenediamine tetraactetic acid
HEPES	hydroethylpiperazine N'-2-ethane sulfonic acid
IEF	isoelectric focusing
IEP	isoelectric point
NP-40	Nonidet P-40
PBS	phosphate-buffered saline
PC	personal computer
PVP	polyvinylpyrrolidone
Rubisco	ribulosebisphosphate carboxylase/oxygenase
SDS	sodium dodecyl sulfate
TCA	trichloroacetic acid
Tris	tris (hydroxymethyl) aminemethane

Chapter 1

General Introduction

1. Senescence in plants

Some authors define senescence as deteriorative changes leading to the death of an organism, organ, tissue or cell (e.g. Thomas and Stoddart, 1980; Woolhouse and Jenkins, 1983). In my opinion, senescence in plants can be better described as an active process leading to a gradual change of functions and eventually death of the plant(parts). The nature of these processes in the case of leaf senescence is described in section 3.

In animals senescence of specific organs can sometimes be observed, for instance during the changes from one larval stage to another (a tadpole loses its tail and external gills before becoming a mature frog), but when maturity is reached only turnover of cells without net growth of the whole organism occurs. Senescence in plants has special features. Deciduous plants may lose their leaves or shoots to overcome a cold or dry period. The polymeric constituents are degraded and their products are transported and stored elsewhere as reserve material for new growth afterwards. In monocarpic plants the death of the whole organism is usually linked to the development of seeds. Degradation products are mobilized and stored as seed reserves. In this way senescence is a developmental process that plays an important role in the strategy of survival of organisms and species in both deciduous and monocarpic plants.

During this process nucleotides, sugars, amino acids and minerals are obtained from the senescing organs and reutilized in the developing plant parts. The rate of protein breakdown, as an essential part in the mobilization of valuable nitrogen-containing macromolecules, is a measure of the speed with which senescence proceeds (e.g. Wittenbach, 1977). In this thesis protein degradation in the first leaf of the monocarpic plant oats is studied as a model for protein mobilization in senescing organs.

2.Leaf age.

Senescence is inseparably linked to development and therefore, like any

other developmental process, genetically determined (Woolhouse 1974). This is illustrated by comparing differences in leaf age between species. From the initiation of a leaf primordium to full lamina expansion can take from as little as a few days in small annual herbs to as long as four years in the case of the cinnamon fern (*Osmunda cinnamomea* L.). Longevity of the expanded blade also varies greatly (Dale, 1982). The leaves of most angiosperms persist only for one season or part of it, but most gymnosperms and many tropical broad-leafed trees, some palms and many ferns have leaves that live for several years. The needles of Scots pine (*Pinus silvestris* L.) are retained for three, sometimes four years. The leaves of the monkey puzzle tree (*Araucaria araucana* Mol. (K.Koch)) may persist for 8 - 10 years or more, and in some palms leaves may be retained for similar periods before they eventually wither and are shed (Dale, 1982). Extraordinary and incomparable to other cormophyta is *Welwitchia mirabilis* Hook.f.. These plants can reach an age of 600 years or more while developing, apart from the two cotyledons, only two basipetally growing leaves that die at the top and keep growing throughout the entire life of the plant (Von Denffer, 1978).

Within species, leaf age of different lines and cultivars may also differ, for example in wheat and soybean (Boyd and Walker, 1972; Crafts-Brandner and Eglis, 1987; See also section 5). Although developing fruits may accelerate and sometimes even induce leaf senescence (e.g.Lindoo and Nood?n, 1977), the initiation of senescence is usually not influenced by the presence or absence of developing fruits (Crafts - Brandner and Eglis, 1987), nor can senescence be delayed indefinitely by removing all developing flowers or fruits.

3. Control of leaf senescence

Woolhouse (1978) has proposed a generalized scheme in which four phases in the life of a leaf are distinguished. According to this view, leaf emergence and expansion constitute phase I, in which synthesis of cellular constituents predominates. Cell organelles, particularly chloroplasts, develop, and the basic complement of proteins is synthesized. In phase II expansion is complete, maturity is reached and the leaf has changed from a primarily importing to a largely exporting organ. It is assumed that in this phase synthesis of some proteins, but not all, continues, and proteins

present are subject to different rates of turnover. Already, translation of chloroplast mRNA's and the content of such proteins as ribulosebisphosphate carboxylase/oxygenase (Rubisco) rapidly decline (Callow, 1974; Thomas and Stoddart, 1980). The gradual loss of some macromolecular constituents and decline in photosynthesis are followed by a more rapid net loss of nucleic acids, proteins and chlorophyll in phase III. At this stage synthesis of most proteins would decline but hydrolytic enzymes might be synthesized *de novo*. Finally, in phase IV, the contents of macromolecular constituents are markedly reduced and cells collapse. In the petiole cell-wall degrading enzymes necessary for abscission may still be synthesized.

During phases II to IV molecules are broken down in a regulated order, starting with chloroplast proteins, through RNA, total protein, chlorophyll, cell wall carbohydrates and DNA. The products of the breakdown processes, nucleotides, amino acids, sugars and minerals are transported either to growing organs, such as young leaves or flowers, or to storage organs, such as seeds, bulbs and tubers. As a result of this internal recycling a minimum of constituents is wasted.

Leaf development can be seen as a program predefined in genetic information. Senescence is just a subroutine which forms an integral part of it and eventually terminates the program in an orderly way. Operation of this senescence subroutine is initiated at the appropriate time of leaf development. The question arises when the initiation of this subroutine takes place. Leaves must reach a certain age before they become competent to senesce. For example, the first leaf of barley grown in continuous light for 7 days or less loses very little chlorophyll when put in the dark; between 7 and 8 days the leaf acquires the ability to senesce in response to darkness (Thomas and Stoddart, 1980). Before initiation, inhibitors of protein synthesis seem to enhance senescence, probably because proteins essential for further development are no longer produced, whereas application of these inhibitors after initiation retards senescence. At some stage during phase I the leaf becomes competent to senesce; when senescence is initiated, synthesis of proteins seems to be necessary for it to proceed.

Leaf senescence may be delayed or even reversed by decapitation or removal of developing leaves or flowers. Avery (1934) described regreening of tobacco leaves after topping of the plant. In contrast, when the leaf is removed from the plant it usually shows accelerated senescence. These observations indicate that other plant parts participate in the regulation of

the rate of development of a particular leaf. Growth-regulating substances have been implicated in this regulation (Thomas and Stoddart, 1980). Cytokinins have been identified as the most general class of senescence-retarding growth regulators (Richmont and Lang, 1957; Varga and Bruinsma, 1973). A wide range of species is also sensitive to the senescence promoter abscisic acid (ABA), and in many cases cytokinins and ABA interact in a competitive manner (Paranjothy and Wareing, 1971; Trevawas, 1972a, b; Millborrow, 1974; Nooden and Leopold, 1980). Exogenous applications of gibberellins or auxins have been reported to influence senescence in a few species (Woolhouse and Jenkins, 1983). Ethylene is considered to be the hormone ultimately responsible for the senescence of excised leaves (Bruinsma, 1981), but there is often no direct relationship between the amount of ethylene applied or liberated by the organ, and the extent of protein and chlorophyll loss (Aharoni and Lieberman, 1979a-c; Gepstein and Thimann, 1981; Woolhouse and Jenkins, 1983).

The rate of senescence is also largely influenced by external factors: light, water, nutrients, pathogens, and environmental stresses. However, the genetically predefined sequence of processes characteristic for senescence stays the same.

In the intact plant neither a decline nor an increase in the endogenous level of one of the known hormones seems to be the primary event in the induction of the senescence subroutine, although such changes may well be part of, or contribute to, a more complex induction system. Not only the endogenous level of a particular hormone, but also the sensitivity of the plant organ to the hormone determines hormone action. The sensitivity may in turn be under the control of (an)other hormone(s) (Thimann, 1980; Bruinsma, 1981; Gepstein and Thimann, 1981).

4.Protein breakdown during senescence

Foliar senescence of both monocots and dicots has been studied in many laboratories. Much work on leaf senescence has been conducted using the primary leaf of oats (*Avena sativa* L.). In darkness, detached leaves start losing protein and chlorophyll within 1-2 days and within the next 2-4 days most of these molecules are degraded (Thimann, 1980). Since the onset of senescence is invariably closely linked to a decrease in protein content, work by the groups of Thimann and Van Loon has concentrated on the possible

role of proteases in the regulation of senescence. Two major proteases were described, one called the acidic protease, with a pH optimum of about 4.5, the other the neutral protease, with an optimum at around pH 7.5 (Martin and Thimann, 1972; Van Loon *et al.*, 1987). The acidic protease is located mainly inside the vacuole, whereas the neutral protease appears to be present in the cytosol (Van der Valk and Van Loon, 1988). Martin and Thimann (1972) concluded that both protease activities increase during senescence. However, Van Loon *et al.* (1987) demonstrated that under non-stress conditions increases in these protease activities do not occur when senescence proceeds. Similar results have been described for rice leaves (Kar and Mishra, 1975) tobacco leaf disks (Anderson and Rowan, 1965; Balz, 1966; Kawashima *et al.*, 1967) and corollas of the morning glory flower (Matile and Winkenbach, 1971). Beevers (1968) and Spencer and Titus (1972) found no correlation between proteolytic activity and protein loss during accelerated senescence of Nasturtium leaves and autumnal senescence of apple leaves, respectively. It can be concluded that there is no relationship between the amount of the major proteases present and the rate of protein breakdown during senescence. Alternative possibilities for the regulation of protein degradation (e.g. decompartmentalization, minor proteases) are discussed in detail by Van der Valk (1987).

During senescence, net protein breakdown is initiated in the chloroplast. Protein degradation precedes the loss of chlorophyll but already involves a reduction in photosynthesis because the major chloroplast protein, Rubisco, is degraded most rapidly (Thomas and Stoddart, 1980). However, Yoshida (1961) observed that in cells of *Elodea*, plasmolyzed under conditions which caused cleavage of the protoplasts, chloroplast breakdown occurred only if the fragments contained a nucleus. Choe and Thimann (1974, 1975, 1977) demonstrated that in isolated chloroplasts protein loss occurs substantially more slowly than in intact tissues. By incubation of isolated chloroplasts in mixtures of enzymes approximating those in the cytoplasm, a rate of loss of both chlorophyll and protein similar to that occurring in intact leaves could be induced. Inhibitors of nucleic acid synthesis or of protein synthesis on cytoplasmic rather than on chloroplast ribosomes, retard senescence in various plant species (Takegami and Yoshida, 1975; Thomas, 1976; Thomas and Stoddart, 1980; Yu and Kao, 1981). Taken together, these various lines of evidence provide a strong case that protein breakdown in chloroplasts depends on protein synthesis in the cytoplasm. However, the nature of the synthesized proteins is not known.

It is questionable as to how far senescence in detached leaves mimics the situation in the intact plant. Although some studies have been made with leaves attached to the plant, usually either floating leaf disks or leaf segments have been used. A detached leaf cannot exchange metabolites, plant hormones and other possibly essential compounds with other plant parts. Furthermore, the wounding due to cutting could well interfere with the changes due to senescence proper.

Giridhar and Thimann (1985) showed that different methods of wounding, for example, subdividing 3 cm long segments of oat leaves into 5 mm subsegments, gently scraping the adaxial surface of the segments with a sharp blade, making transverse linear cuts, or making many small holes with a needle all, resulted in retarded chlorophyll and protein loss. These authors suggested that rapid effects could be due to wound-induced ethylene. The long-term inhibition of protein loss could be due to a wound-induced protease inhibitor (Ryan *et al.*, 1981). Miller and Huffaker (1985) reported the appearance of four endoproteases in detached barley leaves which were not detected in attached controls, and which correlated with a rapid increase of total proteolytic activity to high levels at the leaf base and to lower levels at the top. Apart from changes in protease activity, senescence in attached and detached leaves deviates also in other ways. Thus, Giridhar and Thimann (1985) reported that besides the retention of the existing proteins, several new proteins could be detected in detached leaves that did not occur in attached leaves. Senescence in attached leaves seems not to be under the control of ethylene, perhaps because of root-supplied cytokinins (Bruinsma, 1981), that keep the leaf insensitive to ethylene action (Tetley and Thimann, 1974)

5. Agricultural implications

Cereals, like oats studied in this thesis, display monocarpic senescence: when the grains are filled and start ripening, the whole plant senesces and dies off. The root system regresses already earlier. At the late stage of plant development, when all remaining leaves turn yellow, the leaf proteins are rapidly hydrolyzed. A substantial part of the degradation products formed (e.g. amino acids) is translocated up to the ears (Thayer and Huffaker, 1984), where they constitute a major source for protein synthesis in the developing grains, particularly under dry conditions

(Dalling *et al.*, 1976). The rate at which valuable metabolic products are mobilized is one of the factors determining seed yield and quality. On the one hand, a prolonged phase of active photosynthesis leads to a higher yield of starch. On the other hand, a rapid mobilization of nitrogen during the later phase of seed filling is important to obtain a high protein content. Wheat lines with rapidly yellowing leaves possess a relatively high content of protein in the seeds (Boyd and Walker, 1972). Conversely, spraying the leaves with regulators that retard senescence increases the period of active photosynthesis but impedes mobilization and, at least in soybean, decreases yield both quantitatively and qualitatively (Nooden *et al.*, 1979). To establish physiological criteria for the improvement of agricultural crops, there is a need to better understand leaf senescence, as well as photosynthesis and translocation. When one aims at improving the protein content of the grains by breeding and genetic manipulation, it is crucial to obtain information about the physiology of protein synthesis and degradation in the leaves.

6. The objective of this thesis.

In this thesis it is investigated whether particular proteins are characteristic of certain stages of leaf development and whether specific proteins can be found that are linked to the senescence stage. Protein metabolism is studied by looking into the rate of protein turnover and the factors determining differential turnover, as well as the susceptibility of the proteins from the various developmental stages to hydrolysis by proteases present in the leaves. The aim is to obtain insight into the significance of particularly those proteins that are synthesized late during leaf development and function in an environment in which proteolytic breakdown predominates. By comparing protein turnover *in vivo* with protein degradation in extracts *in vitro*, it can also be assessed as to how far the rate of protein breakdown *in vivo* is determined by direct contact with the protease present or rather results from decompartmentalization of spatially separated substrates and enzymes.

Chapter 2 describes the changes in protein content qualitatively and quantitatively by analyzing protein extracts of leaves at different stages of development using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). The majority of the proteins either disappeared during

senescence or remained detectable at all stages of development. A small number of high-molecular weight proteins appeared during the early stages of senescence and showed an increase in intensity during further development. No proteins characteristic of senescence could be observed. The amount of protein in any spot is the net result of both synthesis and breakdown, which can be determined by labelling with radioactive amino acids. Chapter 3 describes and compares several methods to label intact oat plants. The *de novo* synthesis of proteins at different stages of development, studied by using *in vivo* protein labelling followed by 2D-PAGE, is described in chapter 4. Particularly the newly appearing high-molecular-weight proteins were synthesized at all stages of senescence. Differences in the rate of degradation of different proteins were determined by following the loss of label from prelabelled protein *in vivo* and by autolysis of protein extracts *in vitro*. Chapter 5 describes the protein degradation *in vitro* at pH 5.5 and 7.5, where compartmentalization does not play a role, and *in vivo* where spatial separation of proteases and protein substrates is likely to occur. The differences found are discussed. In chapter 6 these results are compared with the changes in the protein patterns in detached oat leaves subject to accelerated senescence and in the presence of cytokinin or abscisic acid. To analyze the complex protein patterns on 2D-PAGE gels a Pascal program named GELSCAN was developed for use on an IBM-compatible personal computer. This program is described in chapter 7. Finally, in chapter 8 the findings reported in the previous chapters are discussed.

References

Aharoni N, M Lieberman 1979a Patterns of ethylene production in senescing leaves. Plant Physiol 64: 796–800

Aharoni N, M Lieberman 1979b Ethylene as a regulator of senescence in tobacco leaf disks. Plant Physiol 64: 801–804

Aharoni N, M Lieberman 1979c Production and action of ethylene in senescing leaf disks. Effect of indoleacetic acid, kinetin, silver ion, and carbon dioxide. Plant Physiol 64: 805–809

Anderson JW, KS Rowan 1965 Activity of peptidase in tobacco leaf tissue in relation to senescence. Biochem J 97: 741–746

Avery GS 1934 Structual responses to the practice of topping tobacco plants: a study of cell size, cell number, leaf size and veinage of leaves at different levels on the stalk. Bot Gaz 96:314–329

Balz HP 1966 Intrazelluläre Lokalisation und Function von hydrolytischen Enzymen bei Tabak. Planta 70: 207–236

Beevers L 1968 Growth regulator control of senescence in leaf disks of nasturtium (*Tropaeolum majus*). *In* Wightman F, G Settlerfield, Eds, Biochemistry and Physiology of Plant Growth Substances. The Runge Press Ltd Ottawa. pp 1417–1435

Boyd WJR, MG Walker 1972 Variation in chlorophyll A content and stability in wheat leaves. Ann Bot 36: 87–92

Bruinsma 1980 Hormonal regulation of senescence, ageing, fading, and ripening. *In* Lieberman H, ed, Post-harvest Physiology and Crop Preservation. Plenum Press, New York, pp 141–163

Crafts – Brandner SJ, DB Eglis 1987 Sink removal and leaf senescence in soybean. Cultivar effects. Plant Physiol 85: 662–666

Callow JA 1974 Ribosomal RNA, Fraction I protein synthesis, and ribulose diphosphate carboxylase activity in developing and senescing leaves of cucumber. New Phytol 73: 13–20

Choe HT, KV Thimann 1974 The senescence of isolated chloroplasts. Planta 121: 201–213

Choe HT, KV Thimann 1975 The metabolism of oat leaves during senescence III. The senescence of isolated chloroplasts. Plant Physiol 55: 828–838

Choe HT, KV Thimann 1977 The retention of photosynthetic activity by senescing chloroplasts of oat leaves. Planta 135: 101–107

Dale JE 1982 The growth of leaves. Edward Arnold Publishers Ltd.

Dalling MJ, G Boland, JH Wilson 1976 Relation between acid proteinase activity and redistribution of nitrogen during grain development in wheat. Austr J Plant Physiol 3: 721-730

Gepstein S, KV Thimann 1981 The role of ethylene in the senescence of oat leaves. Plant Physiol 68: 349-354

Giridhar G, KV Thimann 1985 Interaction between senescence and wounding in oat leaves. Plant Physiol 78: 29-33

Kar M, D Mishra 1975 Inorganic pyrophosphate actvitity during rice leaf senescence. Can J Bot 53:503-511

Kawashima N, A Imai, E Tamaki 1967 Studies on protein metabolism in higher plants. IV Changes in protein components in detached leaves of tobacco. Plant Cell Physiol 8: 595-604

Lindoo SJ, LD Nooden 1977 Studies on the behaviour of the senescence signal in Anoka soybeans. plant Physiol 59:1136-114

Martin C, KV Thimann 1972 The role of protein synthesis in the senescence of leaves. I The formation of protease. Plant Physiol 49: 64-71

Matile P, F Winkenbach 1971 Function of lysosomes and lysosomal enzymes in senescing corolla of the morning glory (*Ipomea purpurea*). J Exp Bot 22:759-771

Millborrow BV 1974 The chemistry and physiology of abscisic acid. Ann Rev Plant Physiol 61: 639-643

Miller BL, RC Huffaker 1985 Differential induction of endoproteinases during senescence of attached and detached oat leaves. Plant Physiol 78: 442-446

Nooden LD, GM Kahanak, Y Okatan 1979 Prevention of monocarpic senescence in soybeans with auxin and cytokinin: an antidote for self destruction. Science 206: 841-843

Nooden LD, AC Leopold 1980 Phytohormones and the regulation of senescence and abscission. *In* Letham DS, PB Goodwin, TJV Higgins, Eds, Phytohormones and related compounds: A Comprehensive Treatise Vol 2. Elsevier, Amsterdam, pp 329-370.

Paranjothy K, PF Wareing 1971 The effect of abscisic acid , kinetin and 5-fluorouracil on ribonucleic acid and protein synthesis in senescing radish leaf discs. Planta 99: 112-119

Richmond AE, A Lang 1957 Effect of kinetin on protein content and survival of detached *Xanthium* leaves. Science 125:650-651

Ryan CA, P Bishop, G Pearce, A Darvill, M McNiel, P Albersheim 1981. A sycamore cell-wall polysaccharide and a chemically related tomato leaf

polysaccharide possess similar proteinase inhibitor-inducing activities. Plant Physiol 68: 616-618.

Spencer PW, JS Titus 1972 Biochemical and enzymatic changes in apple leaf tissue during autumnal senescence. Plant Physiol 49: 746-750

Takagami T, K Yoshida 1975 Remarkable retardation of the senescence of tobacco leaf disks by cordycepin, an inhibitor of RNA poly adenylation. Plant Cell Physiol 16: 1163-1166

Tetley RM, KV Thimann 1974 The metabolism of oat leaves during senescence I. Respiration, carbohydrate metabolism, and the action of cytokinins. Plant Physiol 54: 294-303

Thayer SS, RC Huffaker 1984 Vacuolar localization of endoproteinases EP_1 and EP_2 in barley mesophyll cells. Plant Physiology 75: 70-73

Thomas H 1976 Delayed senescence in leaves treated with the protein synthesis inhibitor MDMP. Plant Sci Lett 6: 369-377

Thomas H, JL Stoddart 1980 Leaf senescence. Ann Rev Plant Physiol 31: 83-111

Thimann KV 1980 The senescence of leaves. *In* KV Thimann, Ed, Senescence of plants. CRC press, Boca Raton, FL pp 85-115

Trevawas A 1972a Control of the protein turnover rates in *Lemna minor*. Plant Physiol 49: 47-51

Trevawas A 1972b Determination of the rates of protein synthesis and degradation in *Lemna minor*. Plant Physiol 49: 40-46

Van Loon LC, HCPM Van der Valk, AJ Haverkort, GL Lokhorst 1987 Changes in protease activity in leaves during natural development and accelerated ageing upon detachment. J Plant Physiol 127:339-353

Van der Valk HCPM 1987 Regulation of the development of the first leaf of oats (*Avena sativa* L.). Characterization and subcellular localization of proteases. Ph. D thesis Agricultural University Wageningen.

Van der Valk HCPM, LC Van Loon 1988 Subcellular localization of protease in developing leaves of oats (*Avena sativa* L.). Plant Physiol 87: 536-543

Varga A, J Bruinsma 1973 Effects of different cytokinins on senescence of detached oat leaves. Planta 111: 91-93.

Von Denffer D 1978 In Strasburger, Lehrbuch der Botanik, Teil 1, 31st Edition, Eds. D Gustav Fisher Verlag Stuttgart

Wittenbach VA 1977 Induced senescence of intact wheat seedlings and its reversibility. Plant Physiol 59: 1039-1042

Woolhouse HW, 1974 Longevity and senescence in plants. Sci Prog 61:123-147

Woolhouse HW 1978 Senescence processes in the life cycle of flowering

plants. Bioscience 28:25-31

Woolhouse HW, Jenkins GI 1983 Physiological responses, metabolic changes and regulation during leaf senescence. *In* Dale JE, FY Milthorp, Eds, the growth and function of leaves. Cambridge University. Press. pp 451-487

Yoshida Y 1961 Nuclear control of chloroplast activity in *Elodea* leaf cells. Protoplasma 54: 476-492

Yu SM, CH Kao 1981 Retardation of leaf senescence by inhibitors of RNA and protein synthesis. Physiol Plant 52: 207-210

Chapter 2

Two-dimensional gel electrophoretic analysis of the changes in soluble proteins during development of primary oat (*Avena_sativa* L.) leaves.

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Summary

Two-dimensional polyacrylamide gel electrophoresis was used to follow the pattern of soluble proteins in primary oat (*Avena sativa* L.) leaves from 7 to 37 days of plant development. Phenol-soluble protein samples were separated in up to 500 spots by isoelectric focusing between pH 5 and 8 in the first dimension and 12.5% SDS slab gel electrophoresis in the second dimension, as visualized by silver staining. By immunoblotting the two subunits of ribulosebisphosphate carboxylase were identified. 122 protein spots were further characterized by their presence in isolated chloroplasts.

Upon comparison, three classes of proteins could be distinguished: spots originally present but disappearing during development below the limit of detection (disappearing spots); spots originally absent which become apparent and remain throughout leaf development (appearing spots); spots originally present, which do not undergo substantial changes during development (persisting spots). Major changes occurred between 7 and 12 days after sowing, when the primary leaves rapidly expanded to their final length. During this period, 39 mostly low-molecular-weight polypeptides disappeared and 34 mostly high-molecular-weight spots became apparent. Of these, 16 and 13, respectively, were associated with chloroplasts. During the subsequent loss of protein and chlorophyll, indicative of senescence, the number of spots decreased gradually without new polypeptides becoming apparent, but 75% of the total number was still detectable in yellow, 37 days-old leaves. About half of the disappearing spots were associated with leaf expansion. No polypeptides were detected that were present exclusively in the later stages of leaf development: maturity, senescence, and final collapse.

Introduction

Woolhouse (1978) has proposed a generalized scheme in which four phases in the life of a leaf are distinguished. According to this view, leaf emergence and expansion constitute phase I, in which synthesis of cellular constituents predominates. Cell organelles, particularly chloroplasts, develop, and the basic complement of proteins is synthesized. In phase II expansion is complete, maturity is reached and the leaf has changed from a primarily importing to a largely exporting organ. It is likely that in this phase synthesis of most proteins, but not all, continues, and proteins present are subject to different rates of turnover. Already, synthesis of ribulosebisphosphate carboxylase/oxygenase (Rubisco) rapidly declines as a result of the loss of protein-synthesizing capacity in chloroplasts (Callow, 1974; Thomas and Stoddart, 1980). Gradual loss of some macromolecular constituents and decline in photosynthesis are followed by a more rapid net loss of nucleic acids, proteins and chlorophyll in phase III. At this stage synthesis of most proteins would decline but hydrolytic enzymes may be synthesized *de novo*. Finally, in phase IV, the contents of macromolecular constituents are markedly reduced, synthesis of proteins in the leaf lamina ceases, and cells collapse, but cell-wall degrading enzymes may still be synthesized in the veins and petiole.

In the four phases, different functions of the leaf predominate and it may be expected that these are reflected by sequential changes in the protein complement. Protein synthesis is likely to be required for the leaf to enter and complete each of the phases. Notably, cytoplasmic protein synthesis is necessary for macromolecular breakdown to occur during senescence, as to some extent inhibitors of nucleic acid synthesis, but particularly inhibition of eukaryotic protein synthesis are effective in retarding senescence in various plant species (Thomas and Stoddart, 1980; Yu and Kao, 1981). On the other hand, changes in leaf function may also be marked by loss of specific protein components. Thus, chloroplast proteins appear to be lost rapidly during early senescence (Thomas and Stoddart, 1980). Since the nature of the proteins specific to each phase is largely unknown, in a first attempt a survey was made to determine whether such proteins could be identified and characterized. To this end, the constitution of the soluble proteins from primary oat leaves throughout their development was analyzed using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE).

Materials and methods

Plant material

Oat plants (*Avena_sativa* L. cv. Victory) were grown in a greenhouse at a minimum temperature of 20 °C during the daytime and 17 °C at night. Additional illumination was provided by Philips HPI/T 400 W lamps, ensuring a minimum light intensity of 18 $W.m^{-2}$ for 16 h day^{-1}. Seeds were sown in containers of 50x60x10 cm^3 in pot soil at a spacing of ca. 4 cm. The growing seedlings were supported by a frame of wire netting.

To follow the course of development in primary leaves the distal 4.6 cm were harvested from 7 to 37 days after sowing. At day 37 senescence was completed as judged from the leaf appearance.

Preparation of soluble proteins

Except when stated otherwise, operations were performed at 2 °C. Four g fresh weight of 4.5 cm leaf segments were chilled and ground in a mortar in 10 ml extraction buffer (0.1 M Tris-HCl, 10 mM EDTA, 10 mM 2-mercaptoethanol, 0.1 M NaCl, 0.1 % ascorbic acid, pH 7.7). The homogenate was filtered through gauze and the filtrate was centrifuged successively for 15 min at 10,000 *g*, 30 min at 32,000 *g*, and 2 h at 72,000 *g*.

The resulting supernatant was thoroughly mixed with an equal volume of phenol-mix (100 g distilled phenol, 0.1 g 8-hydroxyquinoline, 25 ml chloroform and 30 ml extraction buffer) (Camacho Hendriquez and Sanger, 1982), and the mixture centrifuged for 10 min at 8,500 *g*. To minimize losses, the buffer phase was reextracted with 0.5 vol. of phenol-mix, whereas to the phenol phase 0.5 vol. extraction buffer was added, and the procedure was repeated. The buffer phase resulting from the latter treatment was reextracted again with the second phenol phase. Both phenol phases were combined, 5 vol. 100 % ethanol were added, and the proteins were precipitated overnight at -20 °C. For the electrophoretic analysis, the protein pellet was dissolved in 1 ml isoelectric focusing (IEF) buffer, containing 9.5 M urea (ultrapure; Biorad), 2 % (v/v) Nonidet P-40 (NP-40), 2 % (w/v) Pharmalyte 5-8 (Pharmacia Fine Chemicals AB, Uppsala) and 5 % (v/v) 2-mercaptoethanol (O'Farrell, 1975). The resulting solution was used directly without further treatment.

Preparation of chloroplast proteins

Soluble chloroplast proteins were prepared by lysing chloroplasts prepared from isolated protoplasts. Protoplasts were prepared from the distal parts of the primary leaves from 9-16 days-old-plants as described in detail by Van der Valk (1984). Protoplasts from leaves older than 16 days cannot be reliably obtained (Van der Valk *et al.*, 1987). Based on chlorophyll content, protoplast yield was about 90%. Protoplasts were resuspended in 50 mM HEPES-KOH buffer pH 7.6, containing 0.33 M sorbitol, 10 mM EDTA and 0.1% BSA, and ruptured by passage through a 20 µm nylon net (Edwards *et al.*, 1978). Chloroplasts were purified by centrifugation through a 40% Percoll cushion and washing three times with 50 mM Tricine-HCl buffer pH 7.9, containing 0.33 M sorbitol, as described by Mills and Joy (1980), after which their intactness was checked by phase-contrast microscopy. Only chloroplast suspensions devoid of contaminating organelles and containing more than 80% class A chloroplasts (Hall, 1972) were used for protein preparation. Yields of intact chloroplasts varied between 3 and 10%. Chloroplasts were lysed for 1 h at 4 °C in protein extraction buffer and centrifuged for 10 min at 10,000 *g* to remove membraneous material. Proteins in the supernatant were precipitated in 80% acetone prior to electrophoretic analysis.

Two-dimensional polyacrylamide gel electrophoresis

2D-PAGE was performed essentially according to O'Farrell (1975) with minor modifications, as described by Van Telgen and Van Loon (1984). In the first dimension, IEF extended from pH 5 to 8 and a 12.5 % acrylamide SDS slab gel was used in the second dimension. The use of 10-15% linear gradient gels (Van Telgen and Van Loon, 1984) compressed the area containing the major low-molecular-weight proteins, resulting in a loss of resolution.

To optimize the reproducibility and, hence, the comparison of the patterns from protein samples taken at different times of leaf development, the electrophoretic procedures were rigorously standardized. Samples containing 20 µg of protein were subjected to IEF simultaneously, on gels made from the same stock solutions, in tubes 140 mm long and with a diameter of 2.7 mm for 5200 V. h. Subsequently, the gel rods were placed on a 3.0% stacking gel (20x140x1.5 mm^3) on top of a 120x140x1.5 mm^3 slab gel, both

also made from the same stock solutions, and electrophoresis was conducted for 1 h at 20 mA, followed by 2.25 h at 40 mA.

Gels were stained with silver essentially as described by Morrissey (1981) with minor modifications. Fixation was carried out in 5 % glutaraldehyde instead of 10 % and, after fixation, gels were rinsed at least three times with glass-distilled water before leaving them to soak in a large volume of water (ca. 2 l) overnight. By this procedure, background staining was substantially reduced (Van Telgen and Van Loon, 1984). Notwithstanding the variability in electrophoretic conditions in different runs, very similar patterns were obtained from different batches of plants grown under standard conditions.

Electrotransfer of polypeptides from 2D-PAGE gels to nitrocellulose paper and immunodetection (Immunoblotting)

After second-dimension gel electrophoresis, proteins were electrotransferred for 1 h at 100 V onto nitrocellulose paper (HAHY; 0.45 µm; Millipore S.A., Molsheim, France) in buffer containing 25 mM Tris-HCl pH 8.3, 150 mM glycine and 20 % (v/v) methanol. The nitrocellulose paper was then incubated for 1 h, with gentle shaking, in 50 ml 1 % BSA in phosphate-buffered saline (PBS)-Tween (6.5 mM K_2HPO_4, 1 mM NaH_2PO_4, 135 mM NaCl, 3 mM KCl and 0.5 % (v/v) Tween-20). Fifty µl of an antiserum recognizing both the large and the small subunit of spinach Rubisco (G. Voordouw, personal communication) were added and incubation was continued at room temperature for 2 h with gentle shaking. The paper was washed five times with 100 ml PBS-Tween and 50 µl anti-rabbit-IgG (H+L)-coupled horseradish peroxidase (Nordic, Tilburg, The Netherlands) was added. After incubation for 2 h with gentle shaking the paper was washed with PBS-Tween and stained for peroxidase activity (Hendriks, 1989).

Analysis of the protein patterns.

Stained gels were photographed for permanent records, but analyses were done on transparencies made directly from the gel with a photocopying machine. The latter method allowed a direct comparison of the protein spots present on any two gels by superimposing the two transparencies. Alterna-

tively, gels were subjected to a comparative analysis with the computer program GELSCAN (Klerk and Jespers, 1989). Spots present at each stage of development were included in a composite map, encompassing all protein spots detected in primary oat leaves. These spots were arbitrarily numbered. For each spot the developmental stages in which it was detected were recorded, and on this basis proteins were attributed to one of four classes: class 1: spots originally present but disappearing during development below the limit of detection (disappearing spots); class 2: spots originally absent which become apparent during development but subsequently disappear below the detection limit (appearing and disappearing spots); class 3: spots originally absent which become apparent and remain throughout leaf development (appearing spots); class 4: spots originally present, which do not appear to undergo major change during development (persisting spots). Some class 4 spots were not fully resolved at all developmental stages, for instance when their position was near the edge of the gel or partly hidden by a very large spot. The sum of the proteins of the classes 1, 2, 3 and 4 was designated Σ: the total number of spots present at any time during leaf development.

Determination of soluble protein and chlorophyll content.

Soluble protein content was determined using the method of Bradford (1976) by taking a sample from the proteins dissolved in IEF sample buffer; BSA was used as a standard. Chlorophyll content of leaf segments was determined according to Bruinsma (1963).

Results

The oat leaves completed expansion about 10 days after sowing (Fig. 1). Soluble protein content reached a maximum of 5 mg. g^{-1} fresh weight at 9 days, followed by a maximum chlorophyll content of 1.8 mg. g^{-1} fresh weight at 12 days. Both maxima were followed by rapid (and almost linear) decreases, protein and chlorophyll contents being 0.9 and 0.6 mg. g^{-1} fresh weight, respectively, at 37 days when the leaves started to dry out and necrose (Fig. 1). Based on these parameters, the course of oat leaf develop-

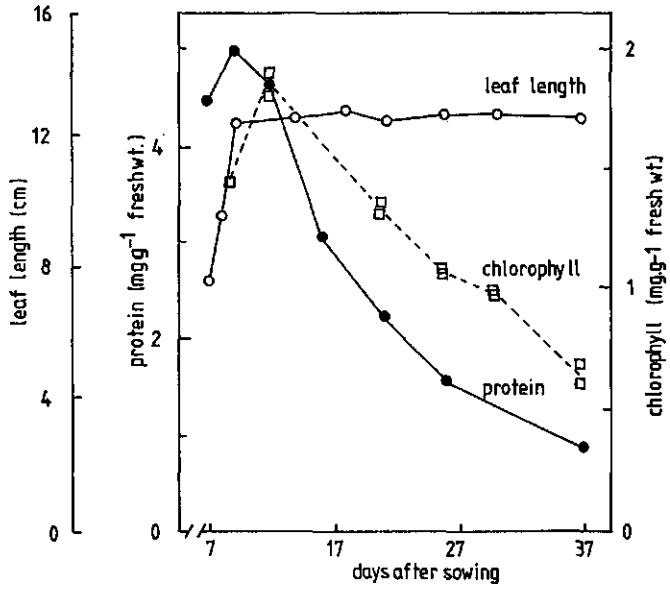


Fig. 1 A representative course of (○) leaf length and changes in (●) soluble-protein and (□) chlorophyll content in primary oat leaves during natural development on intact plants.

ment is characterized by rather short phases of emergence and expansion (phase I; up to 10 days after sowing), and maturity (phase II; 10-14 days), after which a relatively very long period of yellowing (phase III; 14-37 days) preceded total loss of function (phase IV; >37 days). These phases are in accordance with previous observations by Tetley and Thimann (1974) and Van Loon *et al.* (1987).

In preliminary experiments, conditions for the separation of oat leaf proteins by IEF and SDS gel electrophoresis were optimized. IEF extending from pH 3 to 10 revealed that more than 90 % of the detectable proteins had an isoelectric point between 5 and 8, resulting in a compression of the

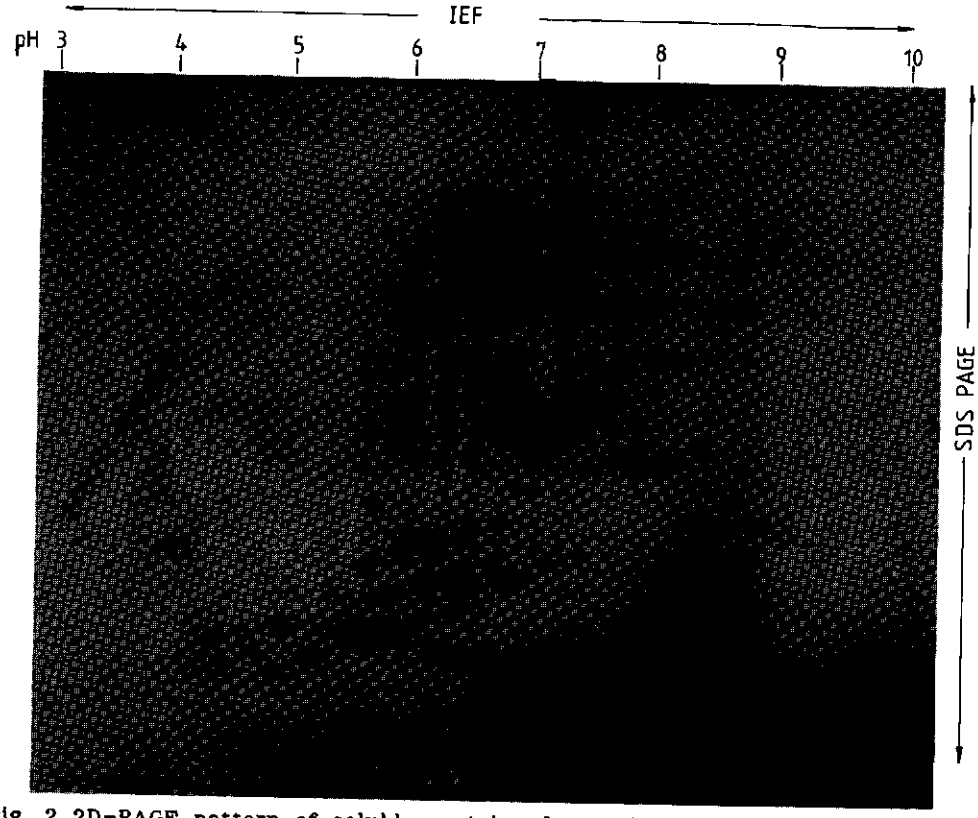
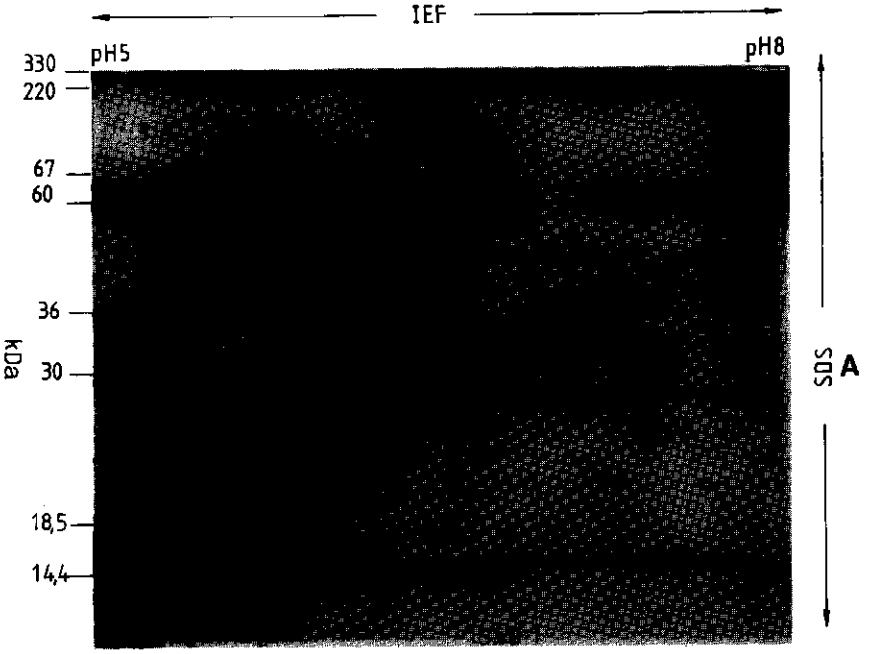


Fig. 2 2D-PAGE pattern of soluble proteins from primary oat leaves from 7-days-old plants. The gradient of isoelectric focusing was from pH 3 to 10. Approximately 50 µg protein was applied to the gel, and the pattern was stained with silver.

protein spots in a small area of the slab gel upon subsequent separation in the second dimension (Fig. 2). Reducing the pH range for IEF from 5 to 8 allowed adequate resolution of these proteins (Fig. 3). Proteins separated by SDS gel electrophoresis in the second dimension displayed molecular weights between 200 and 10 kDa and were well resolved in gels containing 12.5 % acrylamide.

Separation and resolution proved to be critically dependent on the amount of protein applied: too much protein resulted in low-resolution gels with individual spots fused and horizontal background streaking, whereas too small a quantity of protein resulted in faint, undefined spots. For these reasons similar amounts of protein (20 µg) were applied to the gels shown in Fig. 3, rather than amounts corresponding to similar fresh weights of tissue at different stages of development. In this way also a more sensitive detection of proteins present late in leaf development was ensured.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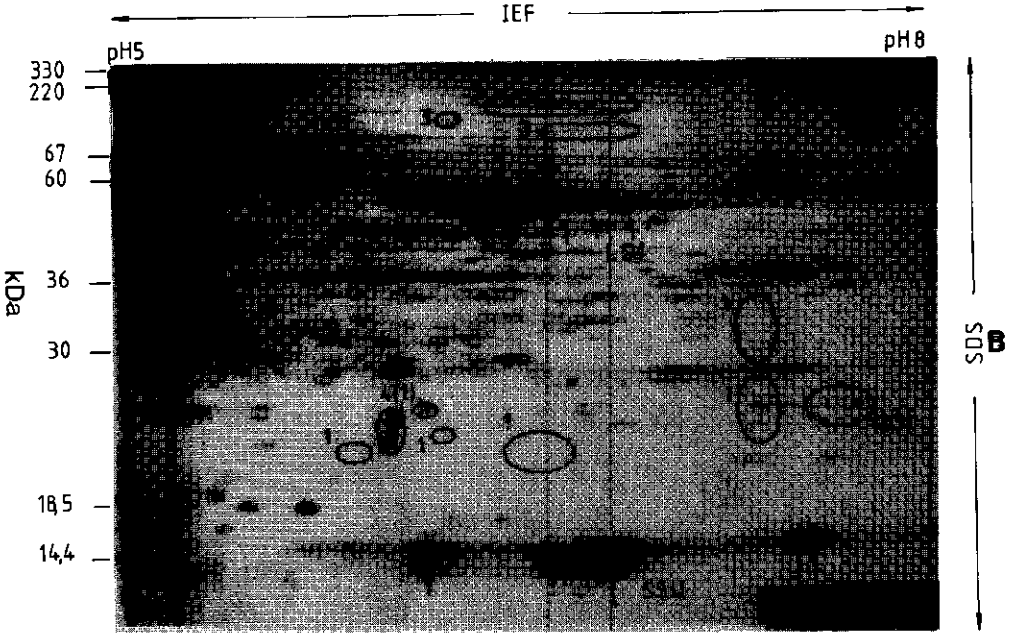
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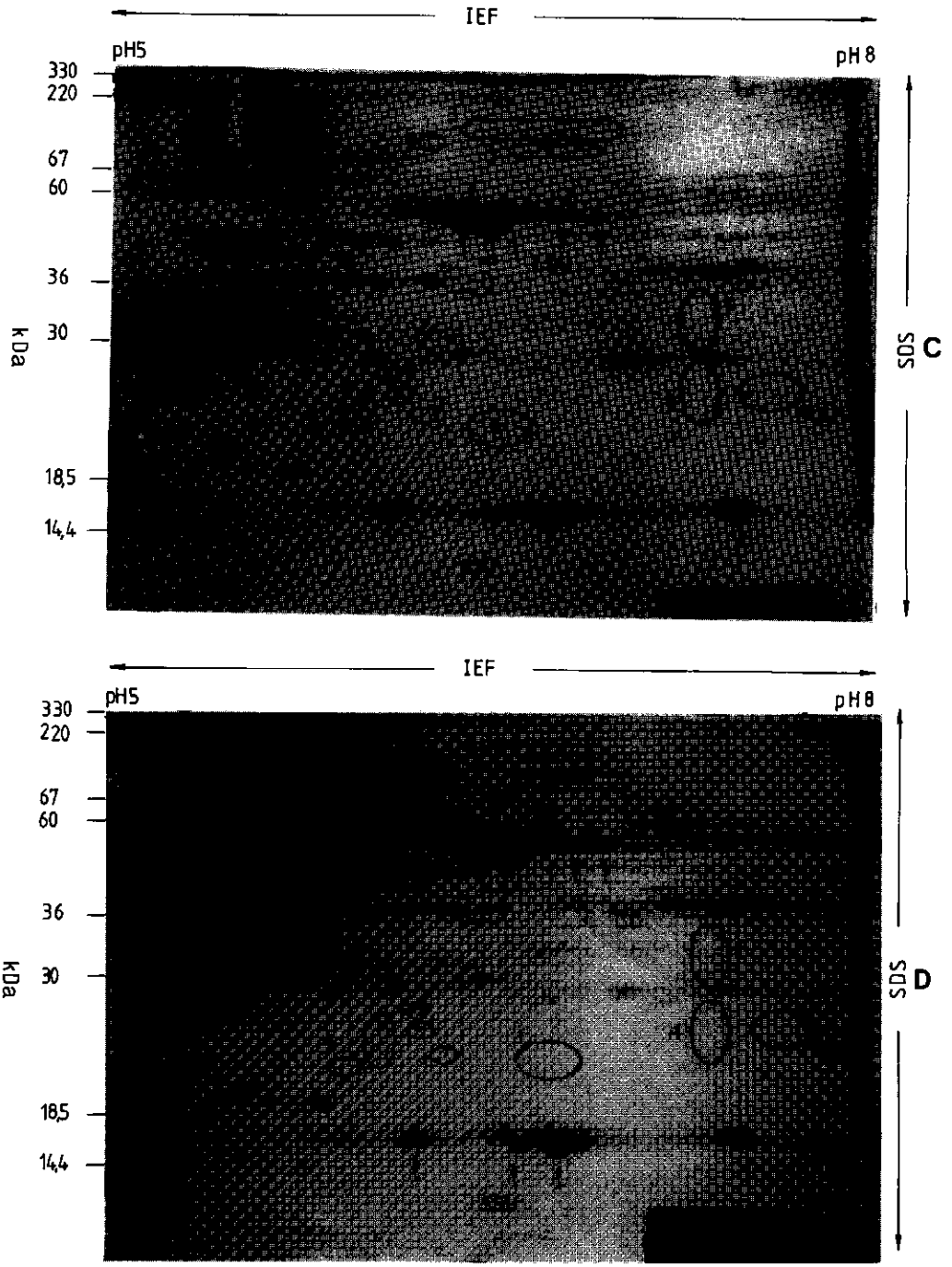
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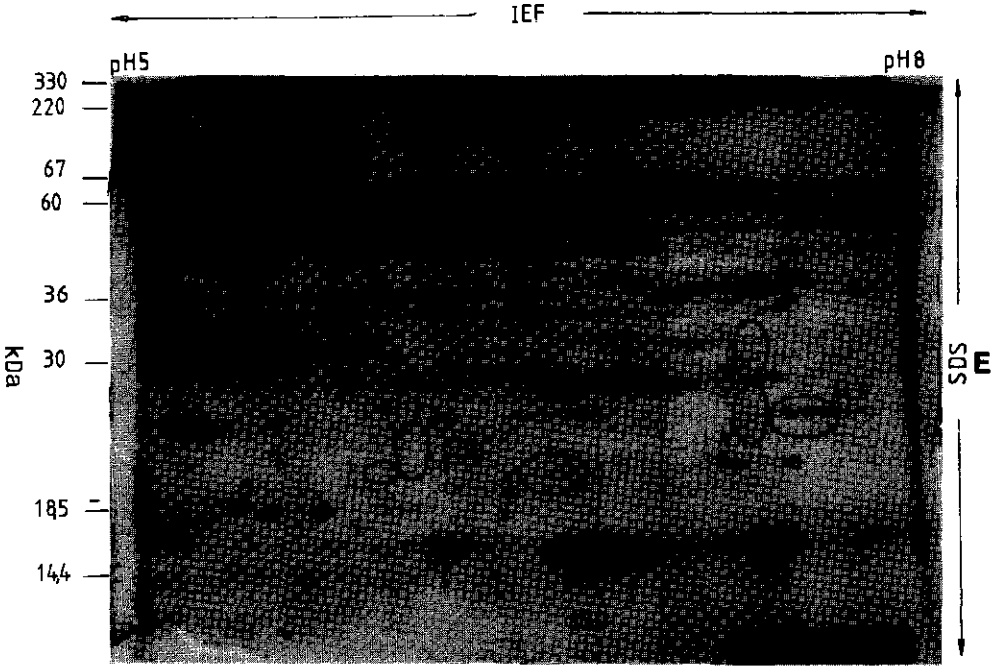


Fig. 3 2D-PAGE patterns of soluble proteins from primary oat leaves from plants (A) 7 days, (B) 12 days, (C) 16 days, (D) 27 days and (E) 37 days after sowing. IEF was from pH 5 to 8. Approximately 20 µg protein was loaded on each gel. Gels were stained with silver. Indicated are some characteristic spots of the classes 1 (disappearing spots) and 3 (appearing spots), as well as a number of class 4 (persisting) spots showing either an increase (4(3)) or a decrease (4(1)) in intensity. The following molecular -weight markers were used: thyroglobulin (330 kDa), ferritin (half unit) (220 kDa), BSA (67 kDa), catalase (60 kDa), lactate dehydrogenase (36 kDa), carbonic anhydrase (30 kDa), ferritin (18.5 kDa), α-lactalbumin (14.4 kDa).

Fig. 3 shows a representative series of soluble-protein patterns at various stages of leaf development. The pattern at 7 days is characteristic of newly expanding leaves, that at 12 days of mature leaves. Those at 16 and 27 days reflect the start and progression of senescence. Finally, at 37 days, leaves were at the verge of drying out. Throughout the sequence, several spots were marked as being characteristic of the classes 1 (disappearing) and 3 (appearing), or representing a number of special class 4 (persisting) spots showing an increase (4(3)) or decrease (4(1)) in intensity and, therefore, a relative increase or decrease with leaf age. Among the intense spots, the large and small subunits of Rubisco were identified by immunoblotting. As shown in Fig. 4, six spots of the large and four of the small subunit of Rubisco were evident. These variants each had similar molecular weights, differing only in net charges. The absence of

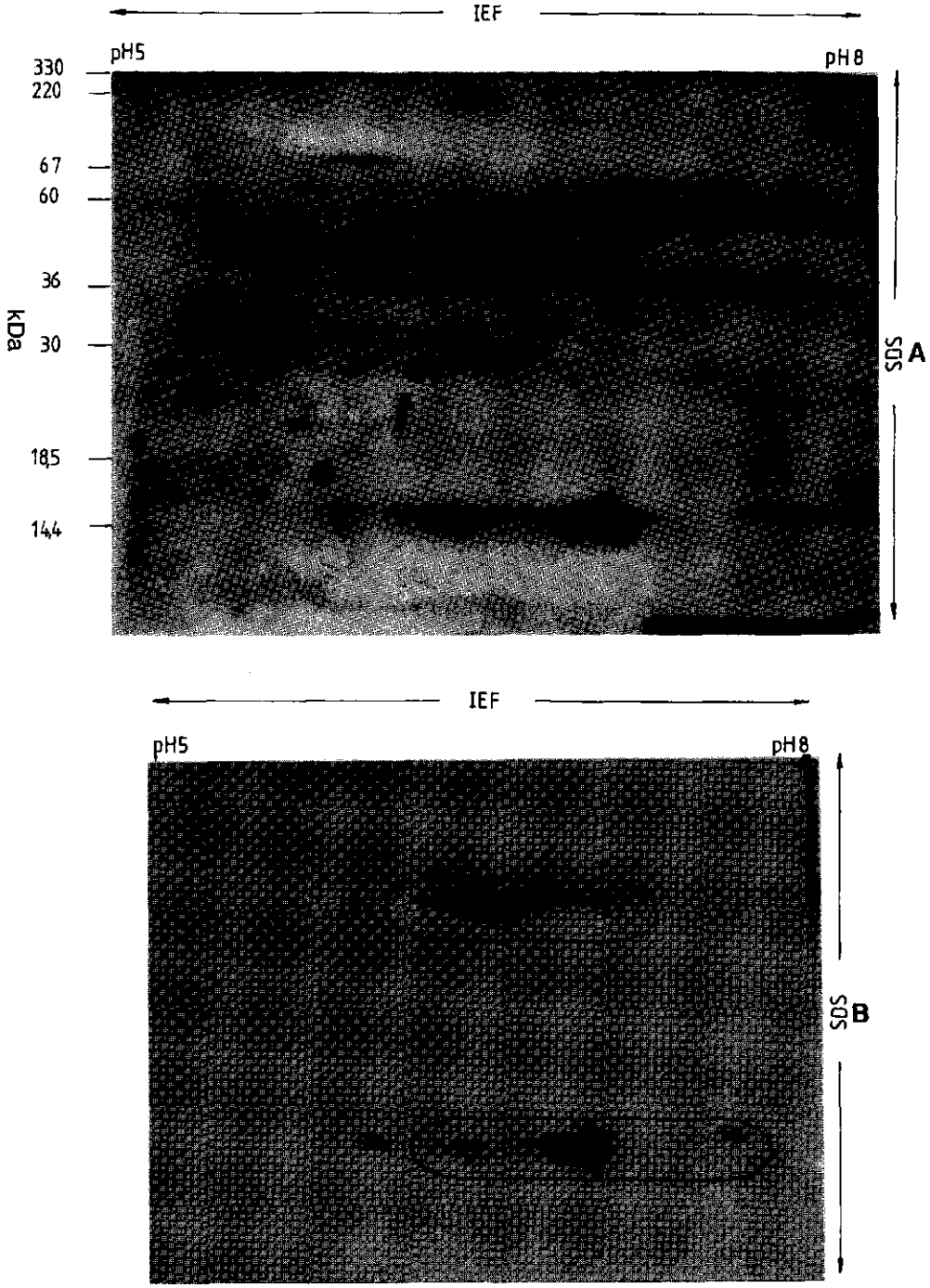


Fig. 4. A silver-stained 2D-PAGE gel pattern (A) and corresponding immunoblot (B) of a soluble protein extract from primary leaves of 9-days-old oat seedlings. A sample of 20 µg protein was subjected to electrophoresis, transferred to nitrocellulose paper, and treated with an antibody recognizing both subunits of Rubisco.

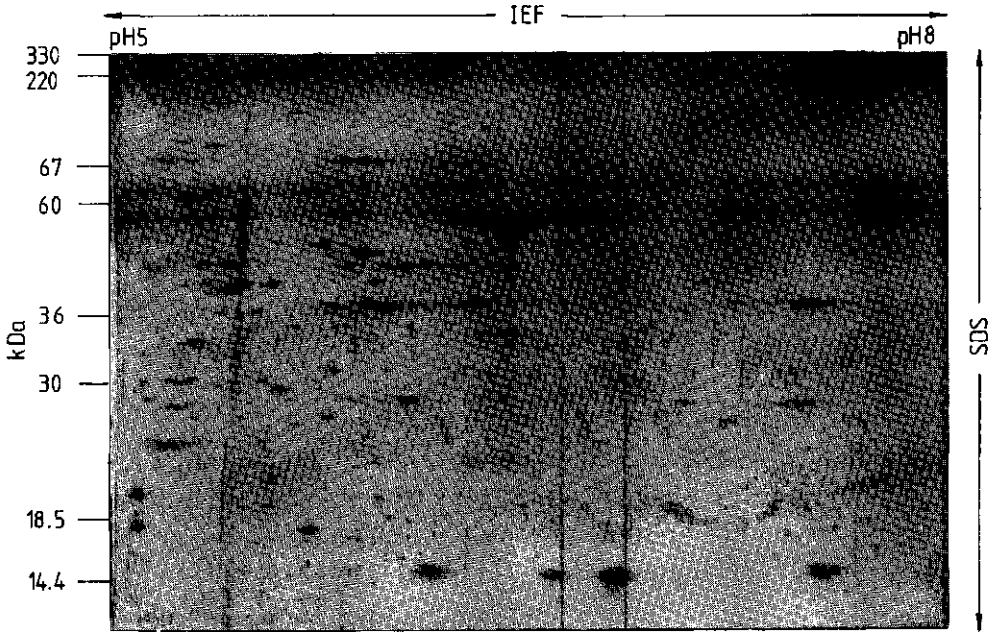


Fig. 5 A silverstained 2D-PAGE pattern of chloroplast soluble proteins from primary leaves of 9-day-old oat seedlings. 20 µg protein was applied and the running conditions were the same as in Fig. 3.

reacting spots with lower molecular weights can be taken as evidence that proteolytic activity during protein preparation was negligible.

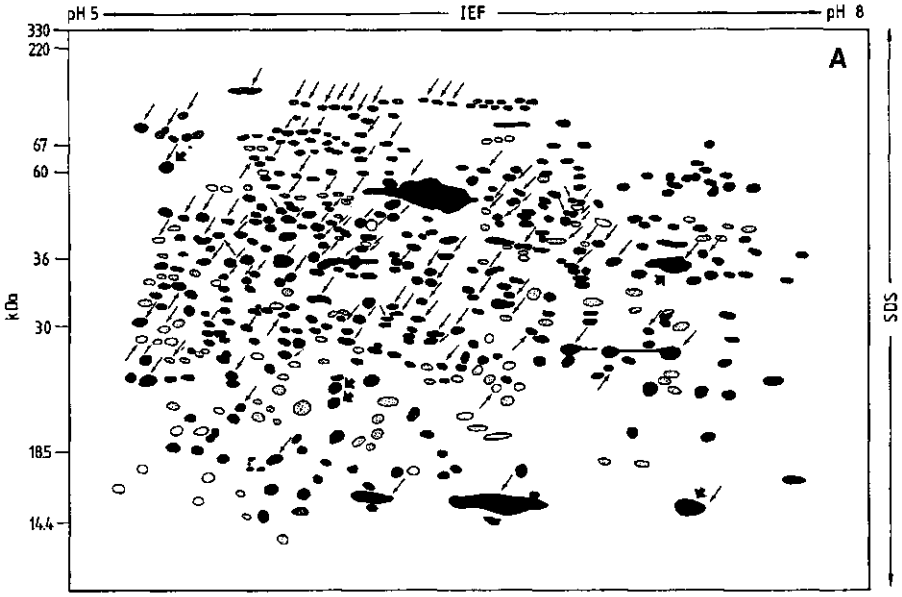
The largest number of spots (427) was detected at 7 days after sowing (Fig. 3A). However, some of the faintest spots were not consistently found in such extracts. At 9 (Fig. 4B) or 12 days after sowing (Fig. 3B) these spots were no longer detected (class 1 spots). Apparently, these spots represent minor proteins that are characteristic of very early stages of development, being present only in emerging leaves. Within this period a similar number of distinct spots appeared (class 3) (compare Fig. 3A and 3B). After the leaf was fully expanded and senescence had started, the overall pattern showed a decrease in the number of spots and an enhanced differentiation between minor and major protein spots: proteins either disappeared or persisted as major spots (Fig. 3C and 3D). This trend continued in the later stages of development and resulted in a well-defined pattern with a majority of large, discrete spots at 37 days (Fig. 3E). The same patterns were apparent when amounts of protein equivalent to equal

amounts of fresh weight of leaf tissue were applied to the gels.

Isolated chloroplasts showed a complex pattern of soluble proteins. As shown in Fig. 5, 158 spots were discernible in patterns from 9-days-old plants. Essentially similar patterns were obtained when chloroplasts were isolated 11, 12, or 16 days after sowing (data not shown). BSA, present in the buffer used for chloroplast isolation, was not detectable, suggesting that contaminating proteins were fully removed during subsequent washing. Of the spots present, 122 were matched to spots seen in the total-soluble-protein pattern (Fig. 4A), identifying these as chloroplast-associated proteins. The high-molecular-weight Class 3 proteins appeared early during leaf development, those with the lower pI were chloroplast-associated. Of the class 1 proteins disappearing during senescence, 16 were chloroplast-associated. Since the same amount of protein was applied to the gels in each case the minor chloroplast proteins not matched to spots in the total-soluble-protein patterns, must have remained below the level of detection in the latter.

Fig. 6 shows diagrams in which the positions and the classes of the individual proteins (Fig. 6A), as well as the number of spots in every class during development are represented (Fig. 6B). By far the most proteins were present throughout leaf development (class 4 spots). From 7 to 12 days after sowing 39 spots, mostly in the low-molecular-weight region of the gel, disappeared (class 1), while the total number of spots remained almost constant: 427 at 7 days and 422 at 12 days. During this period 34 high-molecular-weight protein spots appeared (class 3). After this dynamic period the total number of spots slowly declined, resulting in a still high number of 376 protein spots in yellow-brown, 37-days-old leaves. A small number of spots was recorded only occasionally (Fig. 6A), mainly because they were very weak or obscured by adjacent spots. Consequently, they were not classified, although they were probably class 4 (persisting) spots. There were no indications that these spots were present exclusively at any stage of leaf development. No proteins were detected that appeared and subsequently disappeared between 7 and 37 days (class 2 spots).

Fig. 6 Diagrams of the changes in 2D-PAGE gel patterns presented in Fig.3. (A) A composite map of the locations of the protein spots on 2D gels. black: (class 4); dark gray: (class 3); light gray: (class 1); white: non-classified, occasionally recorded spots. Chloroplast-associated proteins (cf. Fig. 5) are marked with thin arrows; the spots marked with broad arrows were used as marker spots for the alignment of gel patterns from different series (cf. Klerk and Jespers, 1989) (B) Numbers of spots in every class during leaf development: class 1, disappearing spots; class 2, appearing and disappearing spots; class 3, appearing spots; class 4 persisting spots; Σ, the total number of spots (class 1+2+3+4).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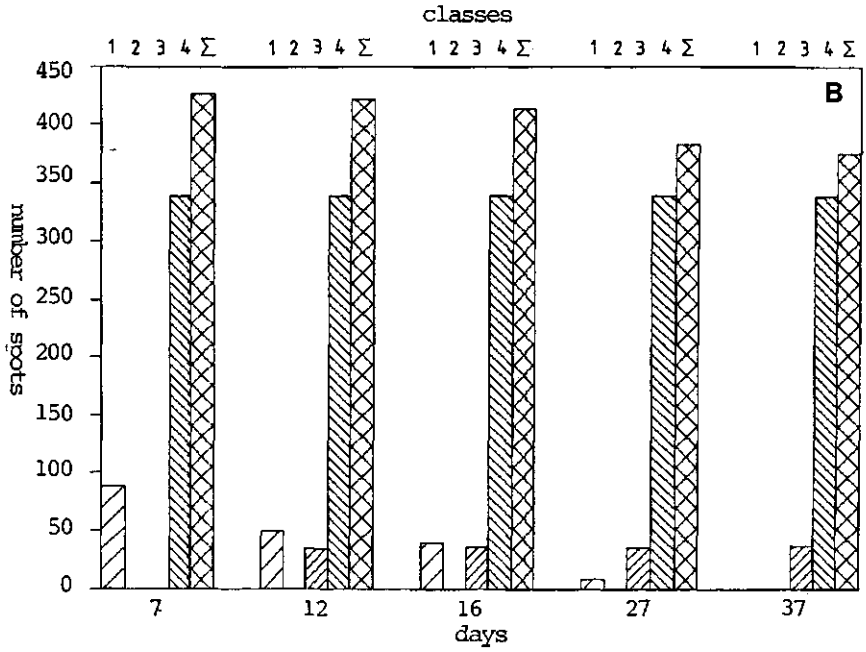
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Discussion

After preparation of centrifuged extracts containing total soluble proteins, phenol extraction was applied to remove nucleic acids, carbohydrates and salts which would interfere in IEF (Van Telgen and Van Loon, 1984). Optimization and standardization of the electrophoretic procedures ensured the obtaining of reproducible protein patterns with excellent resolution. It is unlikely that the number of proteins was affected by protein breakdown during the extraction procedure. The whole operation was performed at 2 °C and the immunoblot of both subunits of Rubisco showed no protein spots of lower molecular weight.

Comparison of the protein patterns from primary oat leaves at different stages of development revealed a total of 493 different protein spots, of which 158 were chloroplast-associated. During leaf development changes in both the number and the relative intensity of protein spots were apparent. By applying the same amount of protein to all the gels, a more sensitive detection of proteins occurring late in leaf development was ensured. In fact, the relatively large number of major spots remaining in senescent leaves was conspicuous. The two subunits of Rubisco were always present. Additionally, major spots representing proteins at (PI, mol. wt. in kDa) 5.2, 62; 7.4, 40; 7.4, 15; 6.0, 22; and 6, 20.5, present in all phases of leaf development, served as markers to align the patterns made at different stages (cf. Klerk and Jespers, 1989).

Based on the major changes observed, the detectable proteins fell into three classes. The majority of spots did not undergo major changes during development. These proteins apparently play a role in the basal metabolism of the leaf (class 4 spots). Class 1 (disappearing) spots obviously represent proteins functioning during leaf growth and are thus characteristic of phase I of leaf development. Essentially all class 3 (appearing) spots had become apparent by day 12, when leaves were fully-grown. These proteins persisted up to a late stage of development comprising at least phases II and III. Several class 1 and class 3 spots were chloroplast-associated. Proteins which might have a transitory role in leaf development (class 2 spots) were not found. Consequently, no proteins characteristic exclusively of either mature or senescing leaves were detected. Neither were any spots observed to appear and increase during the period in which almost linear decreases in protein and chlorophyll contents marked the progression of senescence. Essentially similar conclusions have been reached for pea epi-

cotyls (Schuster and Davies, 1983) and soybean cotyledons (Skadsen and Cherry 1983). Although the earliest and most striking changes associated with leaf senescence occur in chloroplasts, chloroplast-protein patterns remained very similar, even though by 16 days 40% of the protein in the leaf had already been lost. Thus, proteins specifically involved in the senescence process were either absent or their amounts remained below the level of detection. Alternatively, they might be membrane-bound rather than soluble, although soluble cytoplasmic proteins have been postulated to be responsible for the degradation of, e.g., chloroplast proteins in senescing leaves (Choe and Thimann, 1974, 1975, 1977).

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References

Bradford MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254

Bruinsma J 1963 The quantitative analysis of chlorophylls a and b in plant extracts. Photochem Photobiol 2: 241-249

Callow JA 1974 Ribosomal RNA, Fraction I protein synthesis, and ribulose diphosphate carboxylase activity in developing and senescing leaves of cucumber. New Phytol 73: 13-20

Camacho Hendriquez A, Sanger HL 1982 Gelelectrophoretic analysis of phenol-extractable leaf proteins from different viroid-host combinations. Arch Virol 74: 167-180

Choe HT, KV Thimann 1974 The senescence of isolated chloroplasts. Planta

121: 201-213

Choe HT, KV Thimann 1975 The metabolism of oat leaves during senescence III. The senescence of isolated chloroplasts. Plant Physiol 55: 828-838

Choe HT, KV Thimann 1977 The retention of photosynthetic activity by senescing chloroplasts of oat leaves. Planta 135: 101-107

Edwards GE, SP Robinson, NJC Tyler, DA Walker 1978 Photosynthesis by isolated protoplasts, protoplasts extracts and chloroplasts of wheat. Plant Physiol 62: 313-319

Hall DO 1972 Nomenclature for isolated chloroplasts. Nature New Biol 235: 125-126

Hendriks T 1989 Occurence and properties of Petunia peroxidase a. Ph. D thesis Agricultural University Wageningen.

Klerk H, A Jespers 1989 GELSCAN, a Peronal Computer-program to compare protein patterns on two-dimensional polyacrylamide gels. Chapter 7.

Mills WR, KW Joy 1980 A rapid method for isolation of purified, physiologically active chloroplasts, used to study the intracellular distribution of amino acids in pea leaves. Planta 148: 75-83

Morrissey JH 1981 Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. Anal Biochem 117: 307-310

O'Farrell PH 1975 High resolution two-dimensional electrophoresis of proteins J Biol Chem 250: 4007-4021

Shuster AM, E Davies 1983 Ribonucleic acid and protein metabolism in pea epicotyls I. The aging process. Plant Physiol 73: 809-816

Skadsen RW, JM Cherry 1983 Quantitative changes in *in vivo* and *in vitro* protein synthesis in ageing and rejuvenated soybean cotyledons. Plant Physiol 71: 861-868

Tetley RM, KV Thimann 1974 The metabolism of oat leaves during senescence. I Respiration, carbohydrate metabolism, and the action of cytokinins Plant Physiol 54: 294-303

Thomas H, JL Stoddart 1980 Leaf senescence. Ann Rev Plant Physiol 31: 83-111

Van der Valk HCPM 1984 Determination of proteases in isolated washed protoplasts: Inactivation of proteases in cell wall-degrading enzyme mixtures used in protoplast isolation. Plant Sci Letters 36: 201-204

Van der Valk HCPM, LM Plegt, LC Van Loon 1987 Isolation of vacuoles from developing oat leaves. Plant Science 52: 159-167

Van Loon LC, HCPM Van der Valk, AJ Haverkort, GL Lokhorst 1987 Changes in

protease activity in leaves during natural development and accelerated aging upon detachment. J Plant Physiol 127: 339–353

Van Telgen HJ, LC Van Loon 1984 Two-dimensional gel electrophoretic analysis of chromatin-associated proteins from leaves of different species and varieties of *Nicotiana*. J Plant Physiol 118: 285–296

Woolhouse HW 1978 Senescence processes in the life cycle of flowering plants. Bioscience 28: 25–31

Yu SM, CH Kao 1981 Retardation of leaf senescence by inhibitors of RNA and protein synthesis. Physiol Plant 52: 207–210

Chapter 3

Metabolism of proteins during development of the first leaf of oats (*Avena sativa* L.)

I. *In vivo* labelling of the proteins in the first leaf of intact plants.

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Summary

In order to label primary oat leaves *in vivo* for protein turnover studies using two-dimensional polyacrylamide gel electrophoresis, the following methods were tested: growth of seedlings on ^{35}S-sulfate-containing Knop medium, labelling with ^{35}S-methionine by vacuum infiltration of the leaf, injection into the leaf base or into the seed near the embryo, or wiping the surface of the leaf with ethanol and subsequent incubation in the labelled solution. A specific activity of 10^5 dpm / 20 µg of leaf protein minimally necessary to obtain a fluorogram of the gels, was reached only upon the treatment with ethanol, and required not more than 5.5×10^5 Bq of ^{35}S-methionine.

Introduction

In the living organism, proteins are subject to continuous turnover. In developing leaves, synthesis dominates during the phase of leaf expansion, whereas degradation prevails once senescence processes have been initiated (Thomas and Stoddart 1980; Thimann, 1980; Yu and Kao 1981; Dalling, 1986).

It has been assumed that the changes from a primarily importing to a largely exporting organ and the gradual loss of function during senescence in the life of a leaf are associated with differential synthesis and degradation of proteins. To study the turnover of individual proteins during leaf development, their synthesis can be followed by labelling, and their

degradation can be estimated from the loss of label from prelabelled proteins, both after separation by two-dimensional gel electrophoresis (2D-PAGE).

The distal part of the first leaf of oats (*Avena sativa*) has been used by several investigators as a model system to study leaf development (Thimann, 1980; Dalling, 1986). In this organ up to 500 proteins have been distinguished by 2D-PAGE and silver staining (Klerk and Van Loon, 1989). A similar number of spots can be visualized using fluorography after labelling, provided that at least 10^5 dpm of ^{35}S, corresponding to about 20 µg of protein, are applied to the gel. Higher protein loads cause loss of resolution and may lead to streaking and pattern distortion (O'Farrell, 1975; Klerk and Van Loon, 1989), whereas lower amounts of radioactivity result in high backgrounds during prolonged exposure. Thus, the specific activity of the protein fraction should attain at least $5x10^3$ dpm/µg.

Incorporation of ^{35}S into protein can lead to relatively high specific activities, provided that the label is easily taken up by the tissues. For this reason, labelling is usually accomplished on detached leaves, by floating leaf disks or segments on solutions. However, the severing of leaf segments from the rest of the plant engenders both wounding and deprivation of metabolites from other plant parts, and may cause profound changes in protein synthesis and degradation (Watanabe and Imaseki, 1982; Miller and Huffaker, 1985; Giridhar and Thimann, 1985; Klerk *et al.*, 1989). For that reason, a method was sought to obtain sufficient labelling of the proteins in intact leaves on the plant. In this paper, various methods are compared and a simple, efficient procedure is described.

Material and Methods

Plant material.

Oat plants (*Avena sativa* L.) were germinated and grown in a growth cabinet with a photoperiod of 18 h at 25 °C and 17 °C during the night. Light intensity was 12,000 lux from fluorescent tubes (Osram 20R / Cool white and Philips 33RS) supplemented with weak incandescent light (Philips Superlux). Relative humidity was 70%. About 20 seeds were germinated for 2 days in a Petri dish on water-soaked Whatman 3MM paper, after which the seedlings were transferred to a plastic holder (Fig. 1A) within a black-walled glass

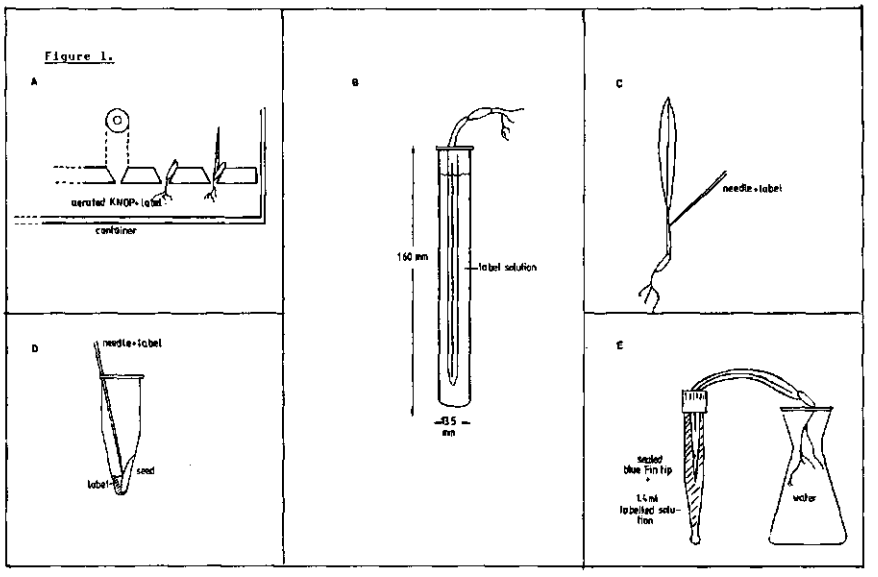


Figure 1. Schematic representation of the methods used to label the proteins of the primary leaf of oats. (See Materials and Methods for details).

container containing 2 l of aerated Knop nutrient solution. The seedlings were grown hydroponically. For plants to be labelled with $^{35}SO_4^{2-}$, $MgCl_2$ was substituted for $MgSO_4$ in the nutrient solution and 12.5 MBq $H_2{}^{35}SO_4$ (spec. act. 200 MBq/mmol) l^{-1} was added. The pH of the medium was maintained between 6.8 and 7.0 with KOH.

Labelling by vacuum infiltration

Hydroponically grown plants were carefully removed from the holder when 7 days old. The first leaves of up to 4 plants were immersed upside down into 15 ml of a solution containing 5.5 MBq of ^{35}S-methionine (spec. act. 44.5 TBq/mmol) in 1mM KCl within a 160 mm long test tube with a diameter of 13.5 mm. The tubes were put in a desiccator and a vacuum of 20 mm Hg was applied for 15 min (Fig. 1B). Subsequently, the plants were carefully put with their roots on wet filter paper. Usually, they were left in the growth cabinet for up to 24 h until harvest.

Labelling by injection into the leaf base

Plants were grown hydroponically as described above. Seven days after sowing the base of the first leaf was injected with 10 µl of a solution containing 14 MBq ^{35}S-methionine in 1mM KCl by using a glass needle and pricking through the coleoptile. The diameter of the needle was adjusted such that when held horizontally the solution was sucked up, whereas the fluid would flow out when it was placed in a vertical position. The needle was clamped at an angle to ensure gradual delivery (Fig. 1C). Under these conditions it took between 0.5 and 2 h for the plant to take up the solution. At intervals, the distribution of the radioactivity applied was followed by separately extracting the coleoptile together with the bases of the first and the second leaf, the proximal, the middle, and the distal part of the first leaf, all of equal length, and the second leaf once it emerged from the coleoptile.

Imbibition and injection of oat seeds

Dry seeds were imbibed for 24 h in an Eppendorf vial in 25 µl of a solution containing various quantities of ^{35}S-methionine (Fig. 1D). Alternatively, the label was applied by injection with a glass needle into the endosperm near the embryo. After treatment, the seeds were transferred directly onto the nutrient solution in the growth cabinet and the seedlings were harvested at regular intervals.

Labelling after pretreatment of the leaf with ethanol

Plantlets grown on nutrient solution for various times were carefully removed from the holder and the distal 4.5 cm of the first leaf was gently brushed three times on both sides with a Kleenex tissue saturated with 96% ethanol. After the ethanol had evaporated, the seedlings were transferred back to the growth cabinet and the wiped part was immersed into 1.4 ml of a solution containing various amounts ^{35}S- methionine. Meanwhile, the roots were kept in tap water (Fig. 1E). Plants were allowed to take up label for a maximum of 24 h and harvested at regular intervals.

Protein extraction

Distal 4.5 cm leaf segments and, in some cases, other parts of the plant, were extracted with extraction buffer containing 0.1 M Tris-HCl, 10 mM EDTA, 10 mM 2-mercaptoethanol, 0.1 M NaCl, 0.1% ascorbic acid, pH 7.7, by grinding in a mortar with a pestle with some seasand and 5% (w/w) PVP at 2 °C (Klerk and Van Loon, 1989). The homogenate was centrifuged for 10 min in an Eppendorf centrifuge. The supernatant was transferred to a clean Eppendorf tube and centrifuged again for 15 min. The supernatant was used directly for determining the incorporation of label and protein content. In some cases the pellet was solubilized in Triton X-100 and likewise used for the measurements. Protein content was determined according to Bradford (1976), using BSA as a standard.

Determination of the incorporation of ^{35}S into protein

The amounts of ^{35}S taken up by the tissue and present in the soluble fraction were determined by adding a 10 µl sample of the extract to 10 ml scintillation fluid and counting by liquid scintillation spectrometry. The incorporation of radioactivity into TCA-insoluble products was measured according to Roberts and Paterson (1973). 10 µl aliquots were spotted on filters pretreated with TCA. Whatman 3MM filter paper was saturated with 10% TCA and 1 mM unlabelled methionine solution, dried at 60 °C, and disks with a diameter of 1 cm were punched out. After spotting the filters were washed successively with ice-cold 10% TCA, boiling 7.5% TCA and ether:ethanol (1:1) before drying at 60 °C. The radioactivity retained on the filters was determined by scintillation spectrometry. Similarly treated filters without sample were used as blanks.

The water-insoluble radioactivity solubilized in Triton X-100 was determined by counting 10 µl samples as described above.

Preparation of soluble proteins

Leaf segments were extracted as described above. Individual segments were extracted with 1.5 ml extraction buffer pH 7.7. Proteins were further purified by phenol extraction and acetone precipitation as described elsewhere

(Klerk and Van Loon, 1989). The precipitate was subsequently washed twice with 80% and 100% acetone, respectively, dried under vacuum, and stored at -20 °C.

Two-dimensional polyacrylamide gel electrophoresis

2D-PAGE was performed essentially according to O'Farrell (1975) with minor modifications, as outlined by Van Telgen and Van Loon (1984). In the first dimension, IEF extended from pH 5 to 8, and a 12.5 % acrylamide SDS slab gel was used in the second dimension. Gels were stained with silver.

Fluorography

Gels were fixed successively for 1 h in 50% methanol, 10% acetic acid, and for 30 min in 5% methanol, 7% acetic acid. The fixed gels were prepared for fluorography by soaking for 2 h in EN^{3}HANCE (New England Nuclear, Postfach 401240, 6072 Dreieich, FRG). Subsequently, they were washed twice for 30 min with ice-cold 5% glycerol, dried at 60 °C, and placed on a preflashed Kodak Xomat AR (Eastman Kodak Company, Rochester, NY 14650, USA) film. The film was exposed for 7 to 14 days.

Results

Growth of oat plants on nutrient solution containing $^{35}SO_4^{2-}$

Fig. 2 shows the result of a representative experiment in which seedlings were grown from 2 days after imbibition onward on nutrient solution containing $^{35}SO_4^{2-}$. After 7 days the first leaf was about 6 to 7 cm long and the distal 4.5 cm contained about $8x10^5$ dpm of water-soluble ^{35}S. This amount increased up to $2x10^6$ dpm upon labelling within the next 8 days (Fig. 2a). However, incorporation into protein, although increasing with time, did not increase beyond $1.7x10^4$ dpm/20µg protein at day 15 (Fig. 2b). Because the specific activity of the ^{35}S in the protein was probably diluted through the incorporation of sulfur-containing amino acids liberated from storage proteins, the endosperm was either partially or totally re-

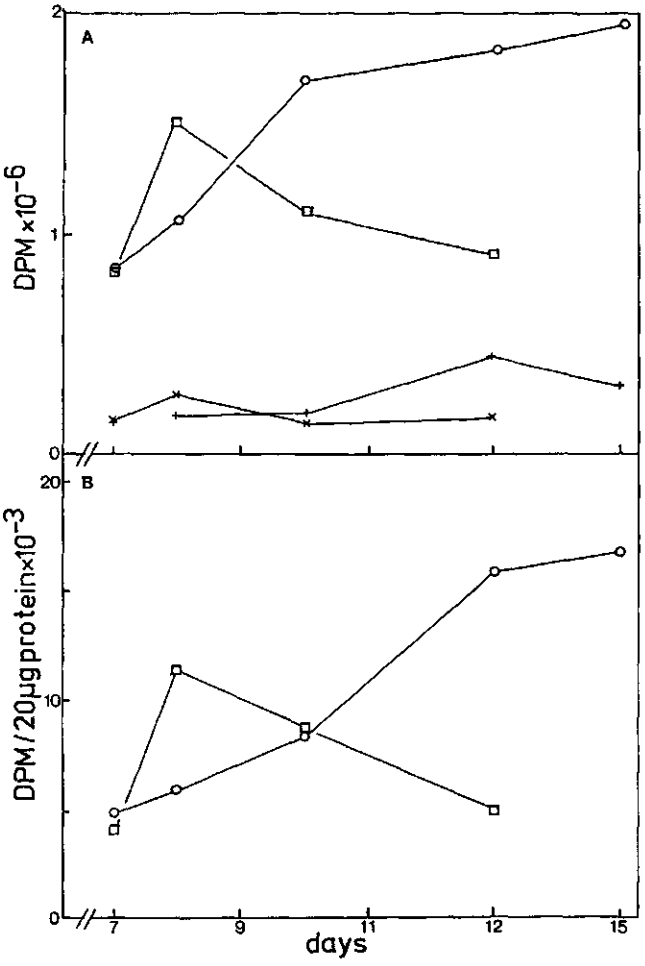


Figure 2. A: Uptake and incorporation of ^{35}S into protein in the distal 4.5 cm of the first leaf of plants growing hydroponically on a nutrient solution (2 l) containing 25 MBq $^{35}SO_4^{2-}$. Radioactivity of the water-soluble (□,○) and water-soluble, TCA insoluble (×,+) fraction, respectively, with or without the endosperm removed at day 7. B: Specific activity of soluble protein when endosperm is removed at day 7 (□) and when endosperm is not removed (○).

moved. Removal of the endosperm before day 7 proved to be deleterious, because growth was impaired and the seedlings died within a few days. Removal of the endosperm at day 7 resulted in increased uptake and incorporation of the label by day 8, but during the subsequent days both water-soluble ^{35}S and labelled protein were lost from the leaves. Removal of half of the endosperm at any time did not affect uptake or incorporation. Thus,

the specific activity required for protein analysis was not attained by this method. Application of larger amounts of radioactivity caused progressive reductions in the growth of the first leaf and retardation of further development of the seedling.

Labelling by vacuum infiltration

Leaves were infiltrated with ^{35}S-methionine *in vacuo*. The volume of the solution taken up by this method varied considerably but was typically in the order of only 6 µl. The methionine taken up by the tissue was rapidly metabolized (Fig. 3). Within 4 h a steady level of 30% had become water-insoluble; this radioactivity was solubilized by Triton X-100 and apparently constituted membrane-bound material. After 7 h, of the 70% water-soluble counts 40% had become TCA-insoluble and, thus, had presumably been incorporated into soluble protein. Nevertheless a level of only about $3x10^3$ dpm/20 µg protein was reached. The limitation of this method appeared to be in the uptake of the label, due to the small volume of solution that can be infiltrated into the leaf.

Injection of ^{35}S methionine into the base of the primary leaf

^{35}S-methionine was applied by injection through the coleoptile into the base of the first leaf. Results are presented in Table 1. Total uptake into the plant parts was about $2x10^6$ dpm, only 0.2% of the radioactivity taken up from the needle. Most of the labelled solution leaked away between the coleoptile and the leaf sheaths and was removed by washing of the material before extraction.

Although throughout 4 days after the application most of the label was recovered in the leaf bases, ^{35}S did accumulate in the first leaf, but almost all of it was retained in the growing proximal and middle parts only. From day 2 onwards, relatively large amounts of label appeared in the emerging second leaf. A specific activity of about $2x10^4$ dpm/20µg protein was attained in the leaf bases within 1 day, in the proximal part of the first leaf after 2 days, and in the second leaf within 2 days. Apparently due to a lack of transport of the label to the middle and distal parts of the first leaf, the specific activity in these parts remained far too low

for this technique to be useful.

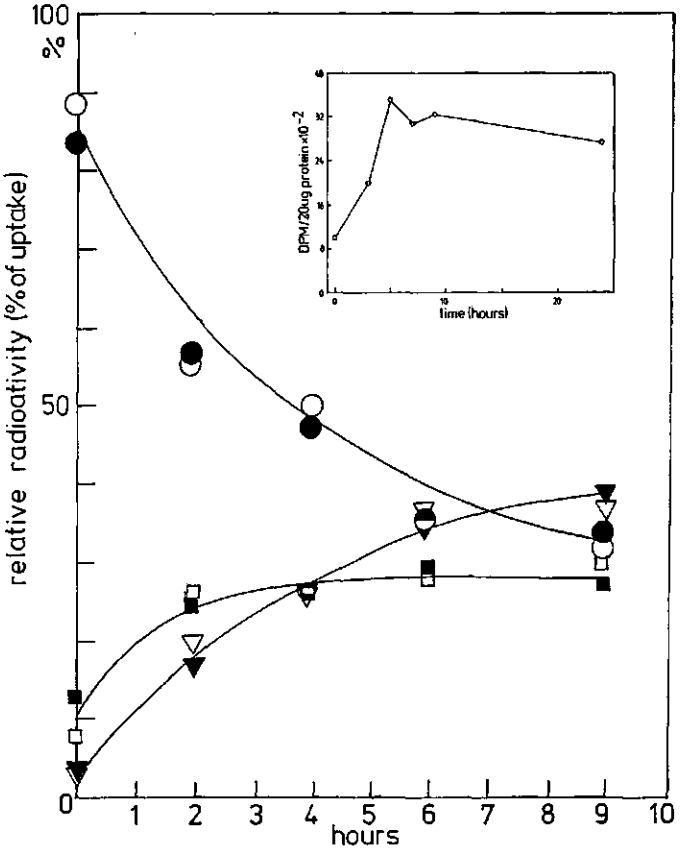


Figure 3. Distribution of radioactivity in percent of total uptake in fractions from the distal 4.5 cm of the first leaf after vacuum infiltration with a solution (14 ml) containing 5.5 MBq ^{35}S-methionine. A: Results from duplicate experiments (open and closed symbols, respectively): ○,● water-soluble, TCA- soluble; □ , ■ water-soluble, TCA-insoluble and ▽,▼ water-insoluble, Triton X-100-soluble fraction. Insert: Specific radioactivity of the soluble-protein fraction.

Imbibition and injection of seeds with ^{35}S-methionine

Application of a solution of ^{35}S-methionine to dry seeds should allow rapid uptake of the label, as well as its availability in early development, when the first leaf emerges. Both imbibition in ^{35}S-methionine-con-

Table 1. Distribution of radioactivity in different plant parts after injection of the leaf base of a 7-day old plant with 14 MBq ^{35}S-methionine. The label was taken up within 2 h. Plant parts were harvested 1, 2 or 4 days after treatment.

Part of plant	Radioactivity (x10^{-3} dpm)								
	Water-soluble			Water-soluble TCA-insoluble			TCA-insoluble per 20 µg protein		
	incub time (days)			incub. time (days)			incub time (days)		
	1	2	4	1	2	4	1	2	4
Coleoptile + Leaf base	1200	1180	910	540	560	530	22.5	34.3	29.0
Proximal third first leaf	850	580	760	100	260	450	5.8	14.5	42.4
Middle third of first leaf	10	89	79	4.1	48	33	4.7	2.2	2.1
Distal third of first leaf	10	33	33	2.1	14	1.8	0.1	0.4	0.5
Second leaf	-[a]	210	290	-	75	100	-	32.7	24.2

[a] Second leaf not yet developed.

Table 2. Effects of the application of ^{35}S-methionine to germinating oat seeds imbibition or glass needle feeding.

Radioactivity applied in the medium (dpm)	Methods Used[a]	Seedling Growth[c]	Specific activity dpm / 20 µg protein
0	+(cold)[b]	++++	0
0	+	--++	n.d.
$8.7x10^3$	-	++++	$5.3x10^2$
$3.0x10^4$	-	-+++	$3.0x10^3$
$1.5x10^5$	-	-+++	$1.2x10^4$
$8.2x10^5$	-	--++	$2.0x10^4$
$3.7x10^6$	-	--++	n.d.[d]
$1.3x10^7$	-	---+	n.d.
$1.3x10^7$	+	---+	n.d.

[a] Seeds were either imbibed in 25 µl water or ^{35}S-methionine containing solution only (-), or additionally fed 25 µl ^{35}S-methionine containing solution (0.5 MBq) through a glass needle (+).
[b] 25µl 1 µmolair of cold methionine were fed as a control.
[c] growth of first leaf (---+, < 1cm long; --++, > 1cm long and < 2cm long; -+++, > 2 cm long; ++++, normal growth (± 13 cm).
[d] n.d. = not determined.

taining water and needle feeding were attempted. Results are summarized in Table 2. Although germination was normal throughout the range of label

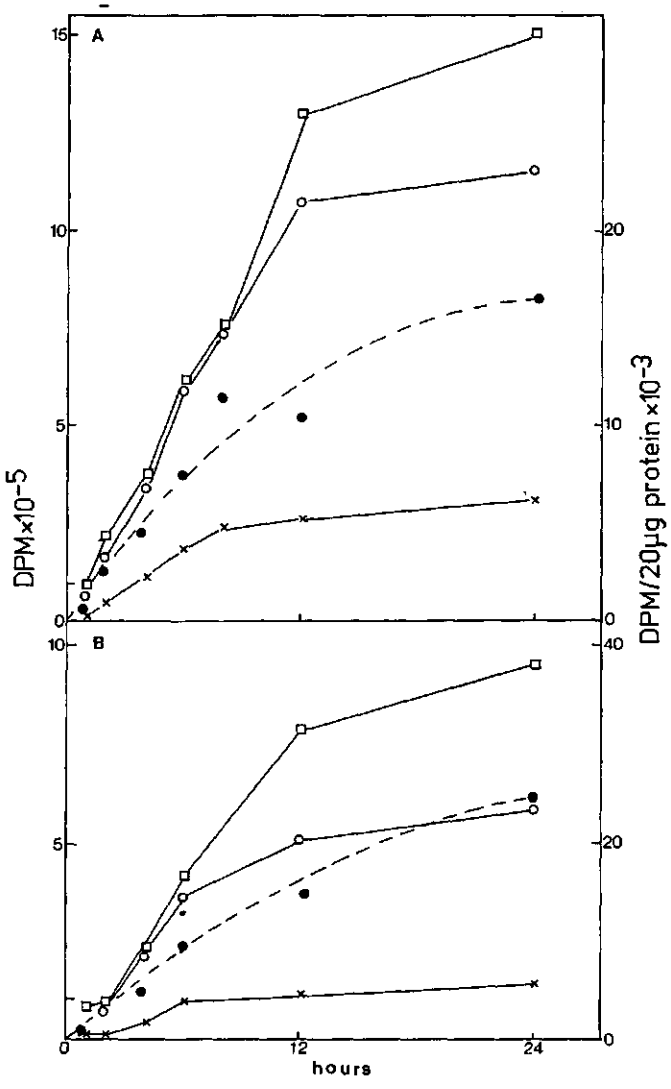


Figure 4. Uptake and incorporation of ^{35}S-methionine in the first leaf of (a) 7-days-oid and (b) 21-days-old plants. The distal 4.5 cm were wiped with ethanol and immersed in a solution containing 39 kBq ml^{-1} and 28 kBq ml^{-1}, respectively; uptake in (□) dpm plant $^{-1}$ and (o) dpm per distal 4.5 cm segment, incorporation in (x) dpm in soluble protein (water-soluble, TCA-insoluble) in the 4.5 cm segment, specific radioactivity of the soluble protein (●) in the 4.5 cm segment. The segments had a protein content of (a) 452 ± 17 (mean ± SE; n=7), and (b) 186 ± 14 µg.

applied. higher radioactivities caused progressive reductions in growth of the first leaf and retardation of further development. Since non-labelled methionine at similar concentrations did not affect growth, these effects must be attributed to radiation damage. A maximum of $2x10^4$ dpm/20µg protein

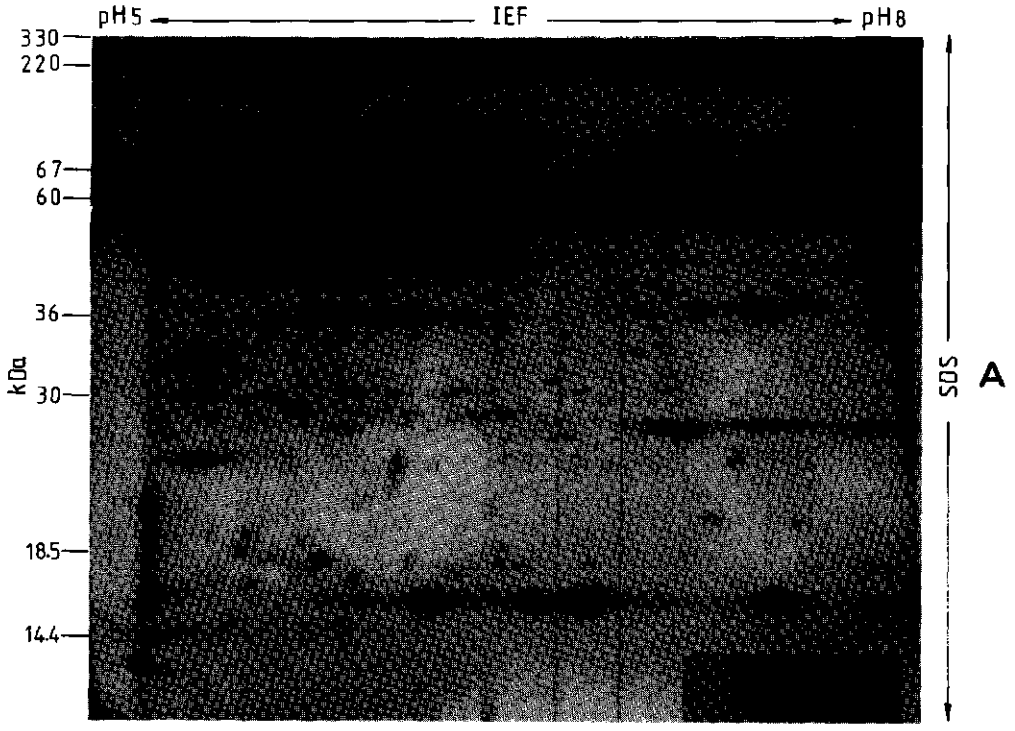
was attained only in the leaf of a plant that stopped growing after reaching a length of 1.8 cm. This method is not applicable because it stops growth and development.

Uptake of ^{35}S-methionine through the epidermis of ethanol-pretreated leaves

Another method for applying the label directly to the leaf tissues would have to overcome the limitations impeding sufficient uptake by vacuum infiltration. To this end, prolonged uptake by immersing the distal part of the leaf into the labelled solution was considered. Little uptake occurred under these conditions but much better results were obtained when the leaf surface was wiped beforehand with ethanol. After this treatment the leaves were slightly darker green as compared to water-wiped controls. Microscopic examination showed that particularly the stomatal cavities were filled with fluid. When ethanol-treated plants were returned to the nutrient solution, growth resumed at the normal rate and no differences with non-treated or water-treated control plants were noticeable, and neither were differences seen in the staining or labelling patterns on 2D-PAGE. Similar treatments with detergents, e.g. 1% Triton X-100 or NP40, led to a slow recovery or even death during subsequent immersion in the labelling solution.

Immersion of the ethanol-treated part of the first leaf in a solution of 1% Neutral Red resulted in a homogeneous accumulation of the dye in the distal 4.5 cm. Thereafter the dye slowly descended through the leaf to the other plant parts. After ethanol pretreatment uptake of label from ^{35}S-methionine-containing solution was linear for up to 12 h after the start of incubation and decreased only slowly thereafter (Fig. 4). Most of the label was retained in the distal 4.5 cm of the leaf; in various experiments 30 ± 1.4% (mean ± SE; n=6) was incorporated into protein, reaching as much as $2x10^4$ dpm/20µg protein when only 55 kBq of ^{35}S-methionine was applied. Similar results were obtained irrespective of the age of the plants. As a result of their lower protein content an even higher specific radioactivity was reached in first leaves from 21-days-old plants than in those of 7-days-old plants.

Figure 5. A silver-stained 2D-PAGE gel and a corresponding fluorogram when 20 µg and 10^5 dpm/20 µg, respectively, of soluble leaf-proteins from 9-days-old plants were applied.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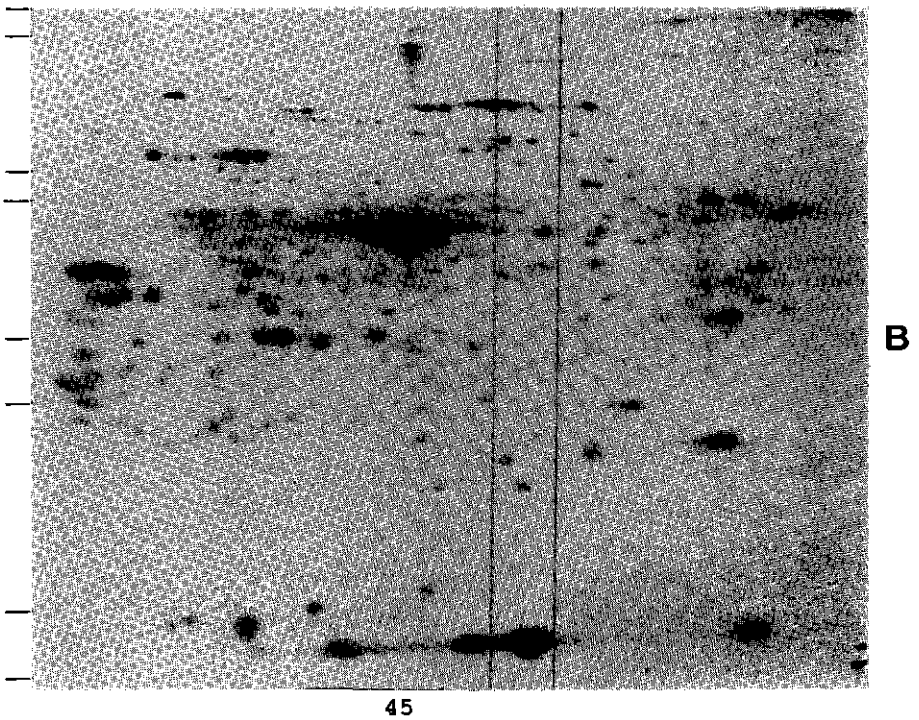
B

Incorporation of ^{35}S into protein was linear with the amount of label applied (Y (Incorporation (dpm/20µg protein)) = 0.18X (Ammount of label applied in Bq) - 0.001; r=0.9999). Since it also proved very reproducible, this method not only allowed us to reach the desired specific activity but also to calculate the precise amount of ^{35}S-methionine to be applied. Thus, for the 7-days-old plants the amount required was 555 kBq. Accordingly, it was possible to adjust the amount of ^{35}S-methionine in the solution in such a way that for plants of any age a specific activity of 10^5 dpm/20µg protein in the first leaf is reached in about 12 h.

A comparison of the labelling and staining methods to visualize the protein patterns indicated that comparable patterns were obtained (Fig. 5). Only relatively minor quantitative differences occur which reflect the differential synthesis of the various proteins. Thus, this method is suitable to follow the fate of individual proteins during leaf development.

Table 3 shows a comparison of the five different methods used. It is clear that uptake by ethanol-treated leaves is by far the most efficient method. Small amounts of radioactivity are sufficient to reach the required specific activity in proteins and no negative effects on growth and development were observed.

Table 3. Summary of the incorporation of ^{35}S into protein using different methods.

Method	Radioactivity applied (dpm)	Radioactivity incorporated (dpm/20µg prot.)	Amount of label required.[a] (dpm)
Growth on $^{35}SO_4^{2-}$	$1.5x10^9$	$1.7x10^4$	$8.8\ 10^9$
Vacuum infiltration of leaf	$3.3x10^8$	$3.0x10^3$	$1.1x10^{10}$
Injection in leaf base	$8.4x10^8$	$0.5x10^3$	$1.6x10^{11}$
Imbibition and injection of seeds	$8.7x10^3$	$5.2x10^2$	$1.6x10^6$
Ethanol treatment of leaf	$3.3x10^6$	$1.0x10^4$	$3.3x10^7$

[a] Extrapolated amount of radioactivity to be applied to reach a specific activity of 10^5 dpm/20µg protein, assuming linearity between supply of label and the extent of incorporation.

Discussion

On the one hand, labelling of proteins in the developing seedling through application of ^{35}S-sulfate or ^{35}S-methionine to the germinating seeds did not prove successful. The label applied to these young plants by the roots

appears to be diluted by cold sulfur-containing amino acids liberated through degradation of seed storage proteins. On the other hand, labelling directly with ^{35}S-methionine through the seed itself, although leading to a relative high incorporation into protein, appeared to be damaging, as growth was severely impaired. Labeling of the leaves with similar doses was tolerated, probably because the radioactivity was applied in less concentrated form and cell divisions had largely ceased. Application of labelled methionine to the leaf bases of the first and emerging second leaf resulted mainly in the labelling of the rapidly growing second leaf. Of the first leaf, only the still growing base was labelled to a significant extent. However to study protein turnover throughout leaf development, a method of labelling is required in which the label is not diverted to competing sinks.

Application of label directly to the 4.5 cm distal part by vacuum infiltration proved very inefficient, because only a small volume of solution entered the leaf. This low uptake may be due partly to the small intercellular space present in oat leaves, and partly to an uptake barrier caused by air pockets in the substomatal cavities. Reducing the surface tension of the leaf with ethanol and incubating the leaves for a longer time in the labelled solution, without vacuum applied, proved to be the only method yielding sufficient incorporation of label into protein. During incubation label accumulated quickly in the leaf and was incorporated into protein in a linear fashion. Thus, the amount of activity to be applied can be accurately calculated. The method is easy to use and only a minimal amount of activity is wasted. Thus, it may also be useful in studies where localized uptake of exogenously applied components is desired.

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References

Bradford MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254.

Dalling MJ 1986 *In* Dalling MJ, Ed, plant proteolytic enzymes Vol. I and II. CRC Press, Inc. Bocta Raton, Florida.

Giridhar G., KV Thimann 1985 Interaction between senescence and wounding in oat leaves. Plant Physiol 78: 29-33

Klerk H, LC Van Loon 1989 Two-dimensional gel electrophoretic changes in soluble proteins during development of primary oat (*Avena sativa L.*) leaves. Chapter 2.

Klerk H, M Rebers and LC Van Loon 1989 Effect of cytokinin and abscisic acid on senescence related changes in soluble proteins in detached oat leaves. Chapter 6.

Miller BL, RC Huffaker 1985 Differential induction of endoproteases during senescence of attached and detached oat leaves. Plant Physiol 78:442-446

O'Farrell, PH 1975 High resolution two-dimensional electrophoresis of proteins J Biol Chem 250:4007-4021

Roberts BE, BM Paterson 1973 Efficient translation of tobacco mosaic virus RNA and rabbit globin gS RNA in a cell - free system from commercial wheat germ. Proc Natl Acad Sci USA 70:2330-2334

Thimann KV 1980 The senescence of leaves. *In* Thimann KV, Ed, Senescence of plants. CRC Press, Bocta Raton, Florida. pp85-115.

Thomas H, JL Stoddart 1980 Leaf senescence. Ann Rev Plant Physiol. 31:83-111

Van Teigen HJ, LC Van Loon 1984 Two-dimensional gel electrophoretic analysis of chromatin-associated proteins from leaves of different species and varieties of Nicotiana. J Plant Physiol 118:285-296

Watanabe A, H Imaseki 1982 Changes in Translatable mRNA in senescing wheat leaves. Plant & Cell Physiol 23(3): 489-497

Yu SM, Kao CH 1981 Retardation of leaf senescence by inhibitors of RNA amd protein synthesis. Physiol Plant 52:207-210

Chapter 4

Metabolism of proteins during development of the first leaf of oats (*Avena sativa* L.).

II Protein synthesis in the first leaf of intact plants

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Summary

Distal segments of the first leaf of intact oat plants were labelled with ^{35}S-methionine at different stages of development. During 12-h labelling similar amounts of label were retained in the segments but incorporation into protein decreased from 28% in 7-days- to 3% in 27-days-old plants. Two-dimensional polyacrylamide gel electrophoretic analysis of the proteins revealed that even at a late stage of senescence a great many proteins are still being synthesized. Synthesis of ribulosebisphosphate carboxylase and the other chloroplast-associated proteins declined more rapidly than general protein synthesis. Two sets of relatively high molecular weight around 67 kDa and an isoelectric point between 6.5 and 6.8 become the most prominently synthesized proteins in older leaves. Since these proteins are hardly visible on silver-stained patterns, they seem to be subject to rapid turnover. A function of these proteins in senescence might explain the requirement of protein synthesis for senescence to proceed.

Introduction

The life of the first leaf of oats (*Avena sativa* L.) extends over a period of about 4 weeks. This relatively short period makes it a suitable object for studying the regulation of leaf senescence (Thimann, 1980; Van Loon *et al.*, 1987). Protein metabolism is crucial to the functioning of the leaf during its development (Woolhouse, 1978) and appears to be a

controlling factor in the rate at which senescence proceeds (Wittenbach, 1977; Thomas and Stoddart, 1980). Protein content in the first leaf of oats is highest shortly after emergence and decreases at an almost linear rate from 8 to about 30 days after sowing (Van Loon *et al.*, 1987; Klerk and Van Loon, 1989). This decrease could result from a gradual cessation of protein synthesis, as well as from an increase in degradation, or both. Application of inhibitors of nucleic acid or protein synthesis retards the loss of protein and chlorophyll in various plant species (Thomas and Stoddart, 1980; Yu and Kao, 1981), indicating that protein synthesis is necessary for senescence to proceed.

Although it was suggested that the requirement for protein synthesis is related to an increase in protease activity (Martin and Thimann, 1972), the major proteases in oat leaves have their highest activities during leaf expansion and only decline when senescence proceeds (Van Loon *et al.*, 1987). Using *in vitro* translation of polyA$^+$-RNA isolated from detached leaf segments subjected to accelerated aging, Malik (1987) was able to distinguish a few polypeptides the synthesis of which appeared to be related to senescence. However, in these short-term experiments, these increases might also be brought about by a wounding response and the additional stresses to which detached leaf segments are subject (cf. Van Loon *et al.*, 1987). Furthermore, the pool of translatable mRNA's may not reliably reflect the proteins synthesized in intact plants. Therefore, an analysis of the proteins synthesized *in vivo* during natural senescence is desirable in order to characterize specific proteins associated with senescence.

A two-dimensional polyacrylamide gel electrophoretic (2D-PAGE) analysis of the soluble proteins throughout the development of primary oat (*Avena sativa* L.) leaves has allowed the identification of up to 500 spots (Klerk and Van Loon, 1989). Major changes occurred between 7 and 12 days after sowing, when leaves rapidly expanded to their final length. During this period several, mostly low-molecular-weight polypeptides disappeared, and 34, mostly high-molecular-weight spots became apparent. During the subsequent loss of protein and chlorophyll indicative of senescence, the number of spots gradually decreased without new polypeptides becoming apparent. No polypeptides were detected that were present exclusively during the onset and/or progression of senescence. It is possible that senescence-related polypeptides never accumulate to levels detectable by conventional protein stains. However, their synthesis may be traced by

labelling and this method is particularly suitable for proteins with a high rate of turnover. In a previous paper (Klerk *et al.*, 1989) a method has been described that allows the labelling of proteins in primary leaves on intact plants. This method has now been used to follow the synthesis of the soluble proteins at different stages of leaf development.

Material and methods

Plant material

Oat plants (*Avena sativa* L.) were grown hydroponically in a growth cabinet as described in detail previously (Klerk *et al.*, 1989). At regular intervals individual plants were selected and both sides of the distal 4.5 cm of the primary leaf were gently rubbed three times with ethanol-saturated Kleenex tissue paper. After the ethanol had evaporated, the treated parts were placed for up to 24 h in vials containing 450 kBq ^{35}S-methionine (29.6 TBq/mmol) (Klerk *et al.*, 1989), in a volume of 1.4 ml. The distal segments were cut off, washed and immediately extracted.

Preparation of soluble proteins and determination of the incorporation of ^{35}S-methionine.

Leaf segments were extracted as described before (Klerk *et al.*, 1989). Each segment was extracted with 1.5 ml extraction buffer (0.1 M Tris-HCl, 10 mM EDTA, 10 mM 2-mercaptoethanol, 0.1 M NaCl and 0.1% ascorbic acid, pH 7.7) by grinding in a mortar with a pestle with some sea sand and 5% (w/w) polyvinylpyrrolidone at 2 ^{0}C. The homogenate was centrifuged for 10 min in an Eppendorf centrifuge. The supernatant was transferred to a clean Eppendorf tube and centrifuged again for 15 min. A 10 µl sample of the supernatant was removed and counted by liquid scintillation spectrometry to determine the total amount of ^{35}S-methionine present in the extract. The amount incorporated into protein was determined after TCA precipitation, as described previously (Klerk *et al.*, 1989). Protein content was determined according to Bradford (1976), using BSA in extraction buffer as a standard.

The proteins present in the supernatant were further purified by phenol extraction and acetone precipitation, as described previously (Klerk and

Van Loon, 1989). The protein precipitate was washed twice with 80% and 100% acetone, respectively, dried under vacuum, and stored at -20°C before analysis by 2D-PAGE.

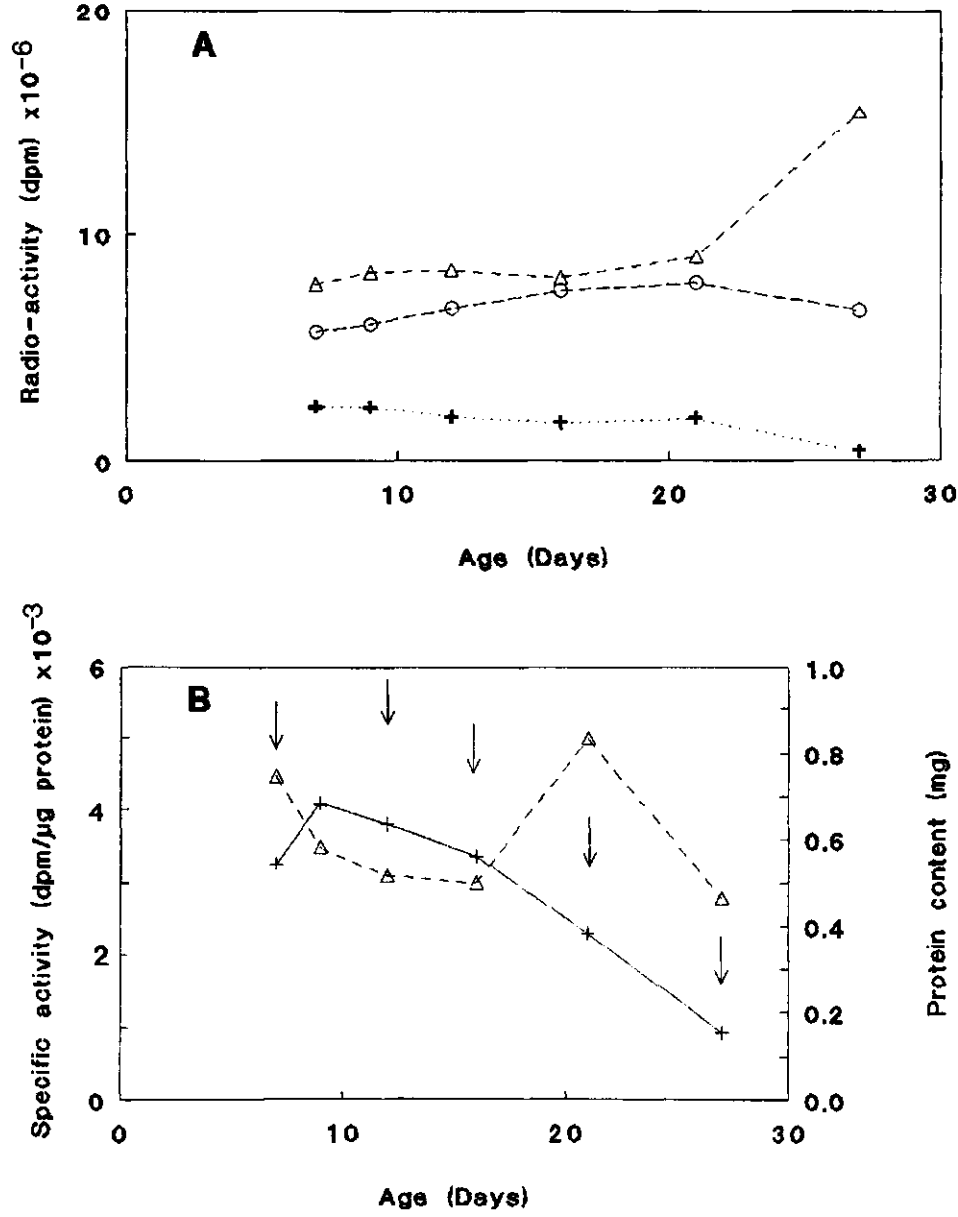


Figure 1. (A) Uptake of ^{35}S-methionine (Δ), amount of label retained (o) and amount of label incorporated into protein (+) in the distal 4.5 cm of primary oat leaves of different ages; (B) Content (+) and specific activity (Δ) of the protein in the distal segment.

Two-dimensional polyacrylamide gel electrophoresis

2D-PAGE was performed as described previously (Klerk and Van Loon, 1989). Similar amounts of protein were applied to all gels. For separation on the basis of isoelectric point in the first dimension, iso-electric focusing (IEF) extended from pH 5 to 8. Separation on the basis of molecular weight in the second dimension was carried out on a 12.5% polyacrylamide slab gel containing 0.1% SDS. Gels were stained with silver according to Van Telgen and Van Loon (1984). Visualization of ^{35}S-methionine-containing protein spots was by fluorography as described previously (Klerk *et al.*, 1989).

Analysis of the protein patterns

Gel patterns were photographed for permanent records, but analyses were done on 1.4 times enlarged photocopies and transparencies made directly from the gel with a reproduction camera. A qualitative comparison of the protein spots present on any two gels was made by computer matching with the program GELSCAN (Klerk and Jespers, 1989).

Results

Uptake and incorporation of ^{35}S-methionine by leaves of different age

After immersion of the distal part of the first leaf in ^{35}S-methionine solution, total uptake of label was almost the same in plants from 7 to 21 days after sowing, but almost twice this amount was taken up by 27-days-old plants (Fig. 1A). Except for this last sample, 75-90% of the label was retained in the distal segment. About the same amount of radioactivity was present in the segment of 27-days-old plants, the remainder having been transported to other plant parts. Percentage incorporation into protein declined from 28% in 7-days- to 3% in 27-days-old plants. In some experiments, uptake of label went through a minimum during the early stage of senescence, around 15 days after sowing, but this variation did not affect the pattern of the proteins synthesized. Labelling for more than 12 h led to additional uptake of label, but this was largely exported from the distal part. Also, incorporation into protein was only slightly increased

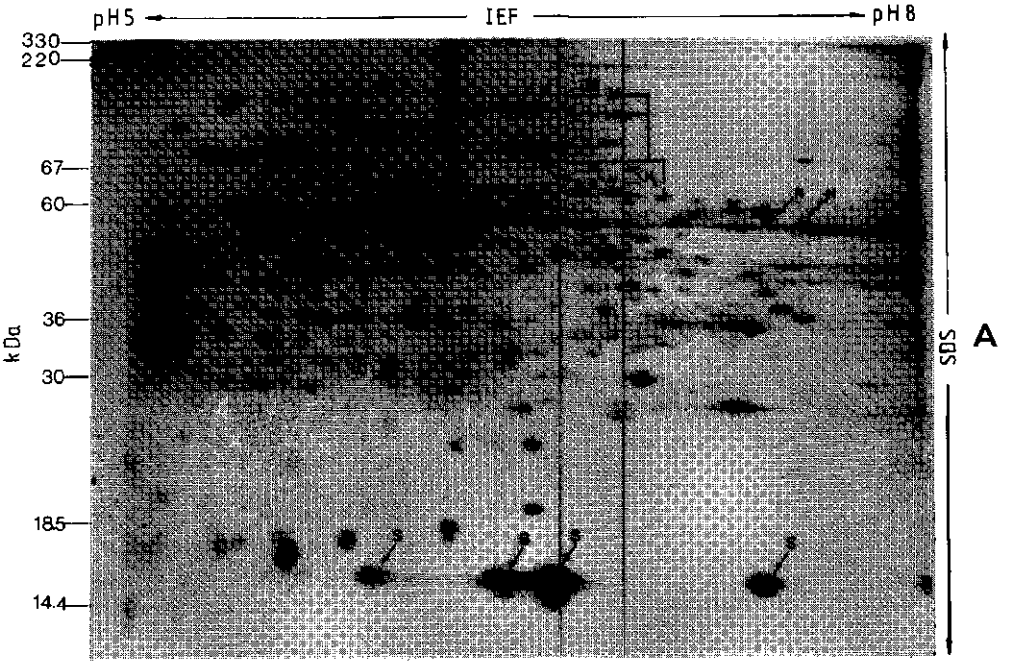
(data not shown; cf. Klerk *et al.*, 1989).

Incorporation of label decreased more strongly than protein content during leaf growth and maturity, but remained steady, or was even slightly increased during senescence, when protein content was rapidly declining (Fig. 1B). Thus, protein specific activity decreased from 7 up to about 16 days, then increased with a maximum around 20 days before finally decreasing again. This apparent increase in protein synthesis during senescence was evident in all experiments and can be explained by a decrease in amino acid pool size (Van Loon *et al.*, 1987), because at this stage rapid translocation of reserves to other plant parts occurs (Thimann *et al.*, 1974).

Analysis of proteins synthesized at different stages of development

Five different stages were selected for analysis of the proteins synthesized (Fig. 1B). At 7 days the first leaf emerges and expands, at 13 days the leaf is mature, at 16 days it shows incipient senescence, at 21 days senescence is well under way and at 27 days the leaf starts to wither. Since high-resolution protein patterns require about 20 µg of protein to be applied to the gel (Klerk and Van Loon, 1989), the patterns of the different stages depicted in Fig. 2 show an overall labelling intensity reflecting the specific activities of the protein (Fig. 1B). Qualitatively the pattern remained quite constant throughout development. A total of around 300 spots could be distinguished and even at a late stage of senescence a great many proteins were still being synthesized. However, during development a few changes stood out.

The large (L) and small (S) subunits of ribulosebisphosphate carboxylase (Rubisco) were the main proteins synthesized in emerging leaves (Fig. 2A), but their synthesis rapidly declined and was no longer detectable from 21 days onwards (Fig. 2B-E). High-molecular-weight proteins, marked H (cf. Klerk and Van Loon, 1989), were synthesized at relatively high levels throughout development. In addition, three proteins, with a molecular weight around 67 kDa and isoelectric points between 6.5 and 6.8 marked N in box I, were synthesized at low rates in 7-days-old plants, but increased in intensity to the most prominent spots labelled in 27-days-old plants. Several more proteins marked N also showed a relative increase in labelling intensity during leaf development. Several proteins previously identified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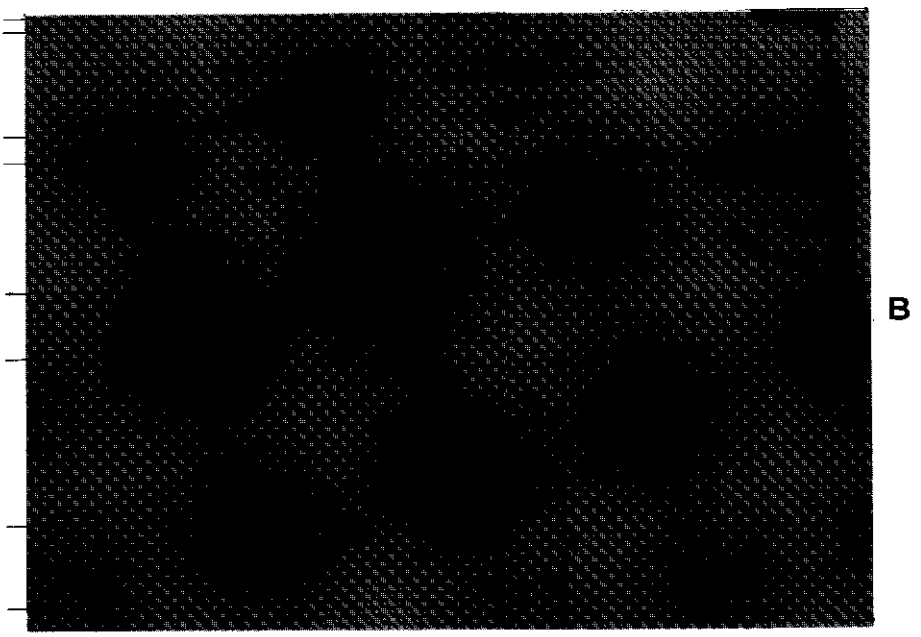
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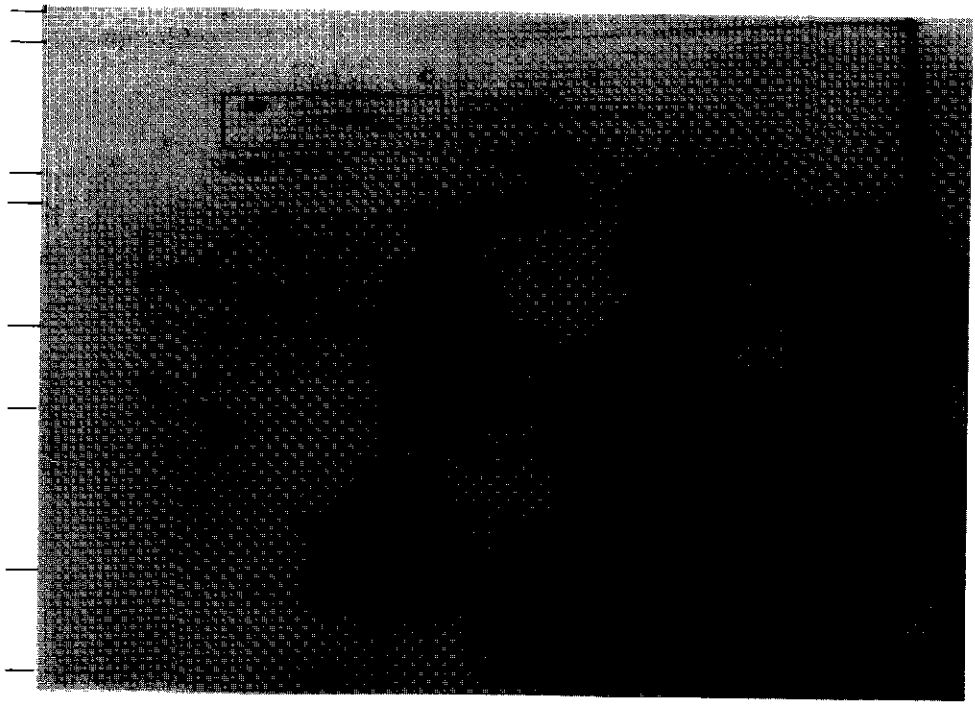

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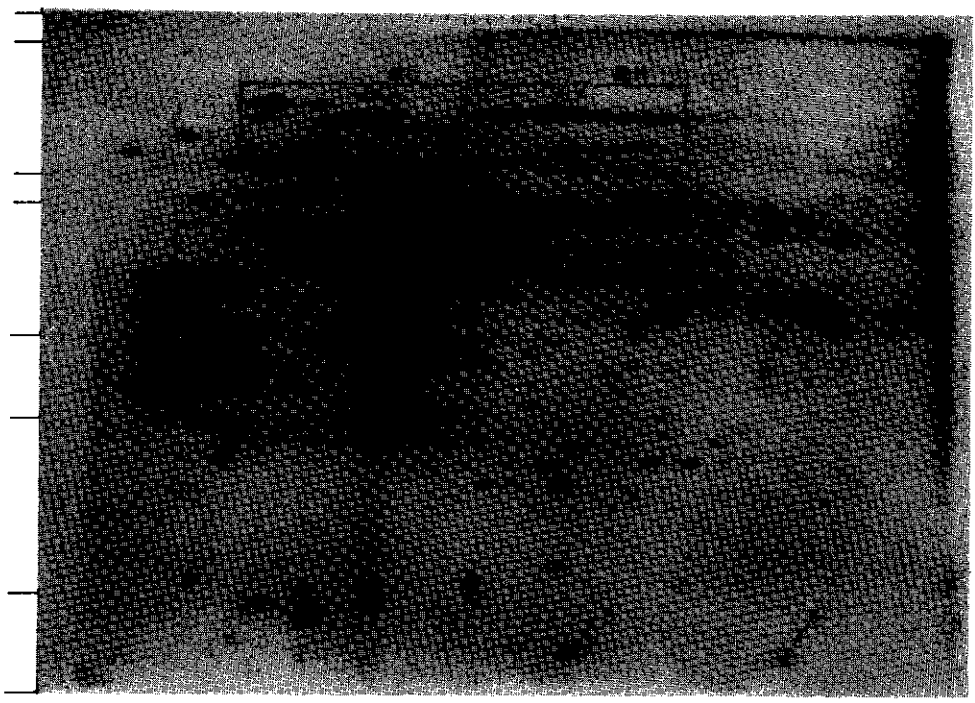

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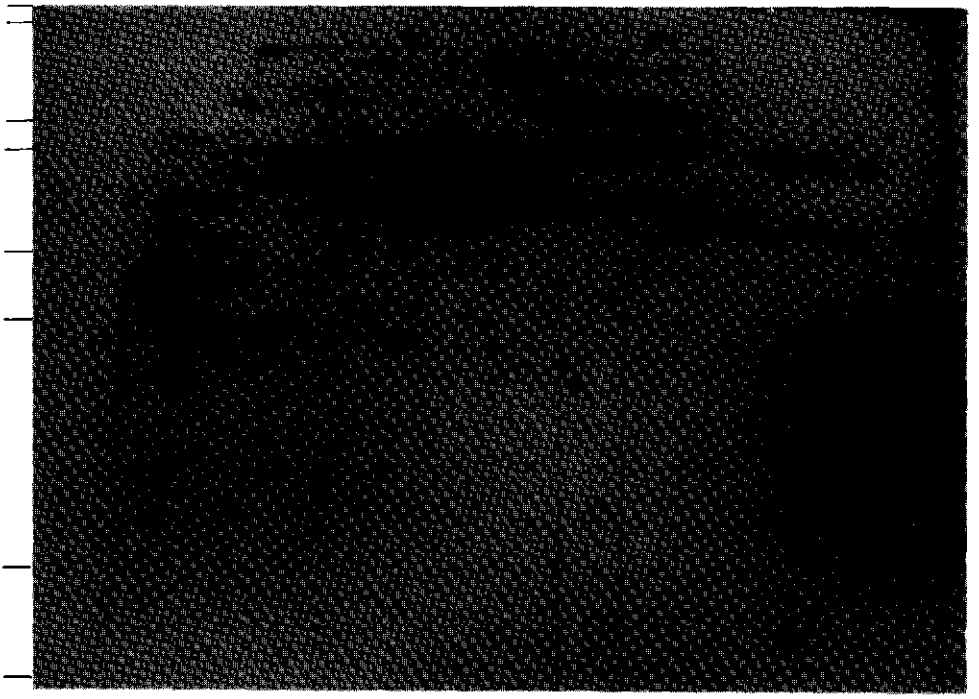


Figure 2. Fluorograms of 2D-PAGE patterns of soluble proteins after a 12-h labelling with ^{35}S-methionine of the distal 4.5 cm of the primary leaves in intact plants (A) 7, (B) 13, (C) 16, (D) 21, and (E) 27 days after sowing. About 20 µg of protein was applied to each gel. The positions of the molecular weight markers are thyroglobulin (330 kDa), ferritin (half unit) (220 kDa), BSA (67 kDa), catalase (60 kDa), lactate dehydrogenase (36 kDa), carbonic anhydrase (30 kDa), ferritin (18.5 kDa), α-lactalbumin (14.4 kDa) are indicated. C=chloroplast-associated polypeptides; CD= disappearing chloroplast-associated polypeptides; D= disappearing spots; H= high-molecular-weight polypeptides; L=Rubisco large subunit; N=senescence-associated polypeptides; N'=newly appearing spots in association with disappearing spots; S=Rubisco small subunit. Spots of particular interest are boxed.

as being chloroplast-associated (C) (Klerk and Van Loon, 1989) were synthesized in decreasing amounts with leaf age. However, even at 27 days labelling was still evident (Fig. 2E). Furthermore, in boxes II and III spots marked CD disappeared, whereas spots marked N' appeared, suggesting a relationship between them.

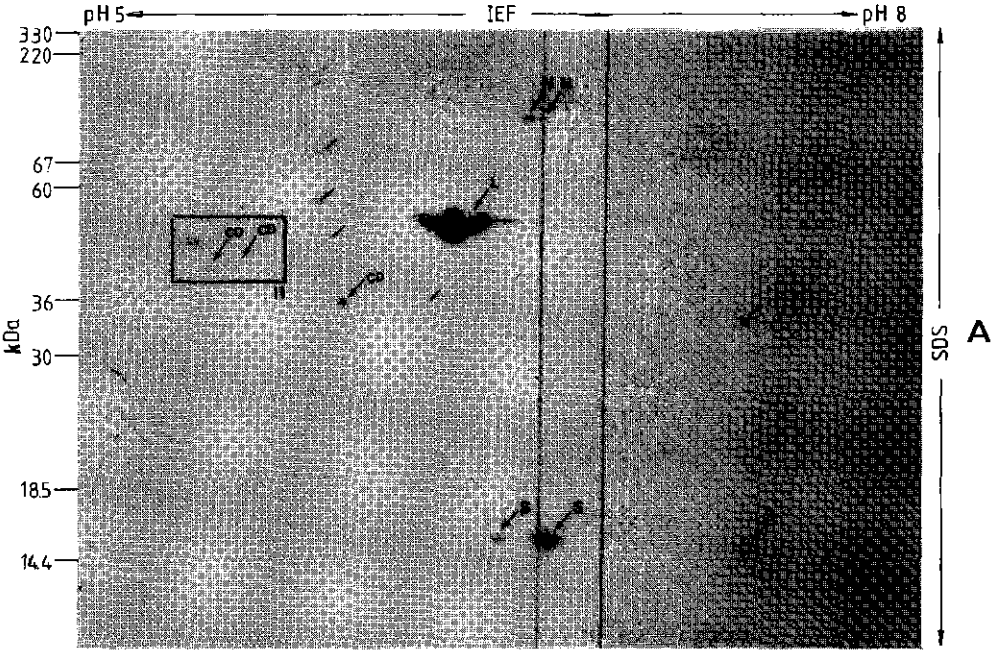
No proteins unique to the stage of senescence were identified, but the preferential synthesis of proteins, marked N, during senescence is suggestive of their involvement in this process. The major changes thus appear to take place early in development. For this reason, the rates of synthesis of the proteins in 7- and 13-days-old plants were further analyzed in order to see whether additional proteins are synthesized at the latter stage, when the plant is fully mature.

Comparison of the proteins synthesized in 7- and 13-days-old plants

A 1-h labelling of leaf segments of 7-days-old plants showed preferential synthesis of the subunits of Rubisco (Fig. 3A). Two high-molecular-weight H proteins were also detectable. Traces of N proteins were first visualized after 6 h and no N' proteins were synthesized. Many other proteins had become apparent after 3 or 6 h of labelling (Fig. 3B, C), indicative of the substantial rate of protein synthesis at this stage.

Fig. 3D-F depict that at 13 days the patterns are quantitatively very different. Traces of Rubisco synthesized were not yet apparent at 6 h labelling, although other chloroplast-associated proteins present in boxes II and III had by then become clearly discernible after 6 h (Fig. 3F). After 1 h only the two H proteins had been synthesized, and at a higher rate than after 7 days (Fig. 3D). More H proteins, notably also those with a lower molecular weights, had been synthesized after 3 h (Fig. 3E) and many additional spots had become apparent. Most of the senescence-associated proteins marked N in Fig 2, although synthesized at these stages, were not yet apparent after 6 h labelling, indicating that their synthesis is much restricted before senescence ensues.

Figure 3. Fluorograms of 2D-PAGE patterns of soluble proteins from the distal 4.5 cm of the primary leaves on intact plants (A-C) 7 and (D-F) 13 days after labelling with ^{35}S-methionine for (A,D) 1, (B,E) 3, and (C,F) 6 h. About 50 µg of protein was applied to each gel. Markings as in Fig 2.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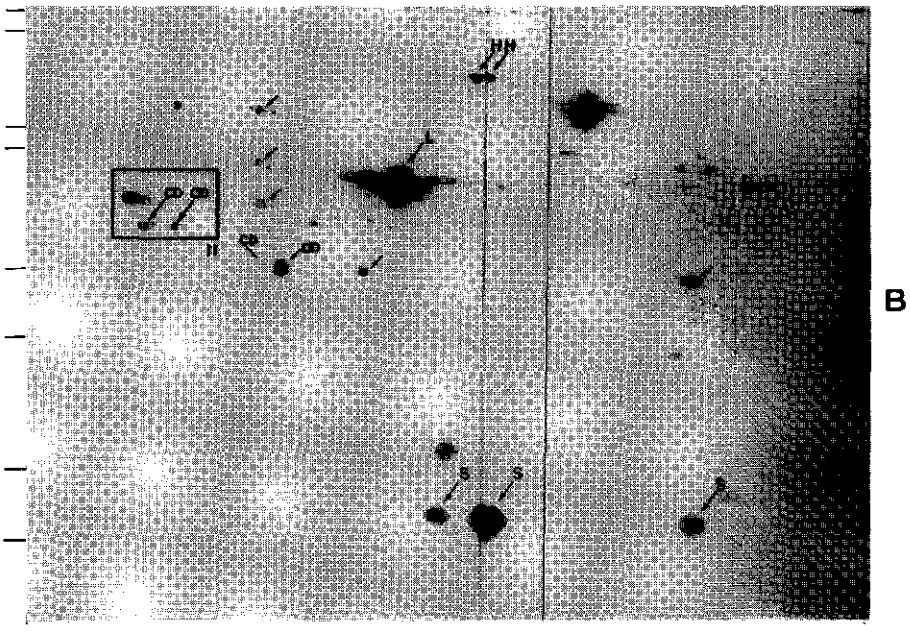
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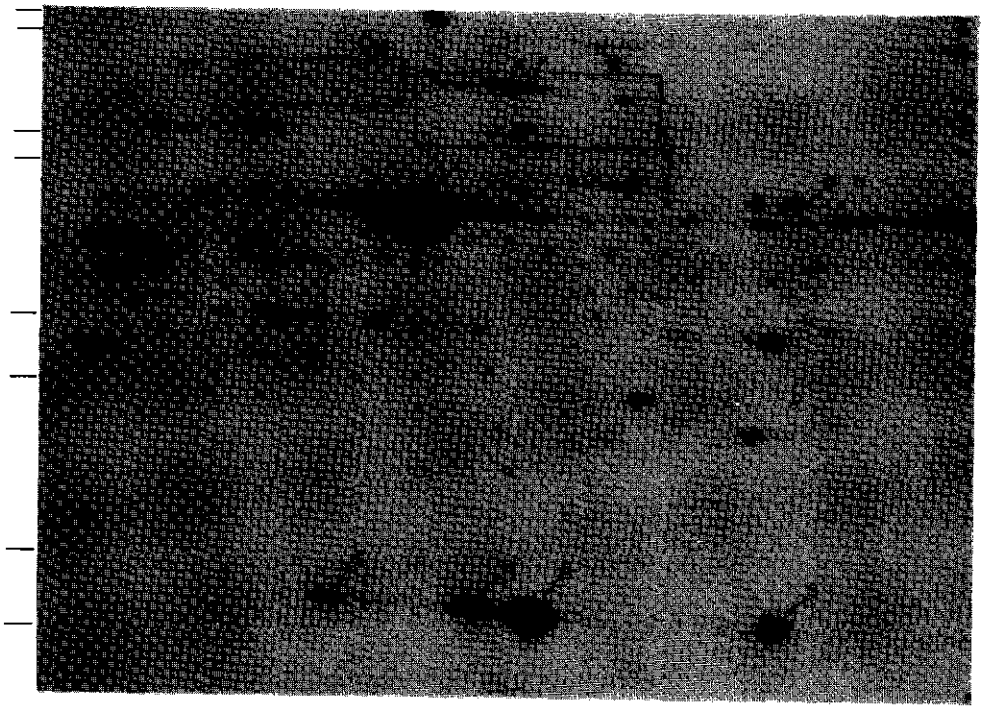

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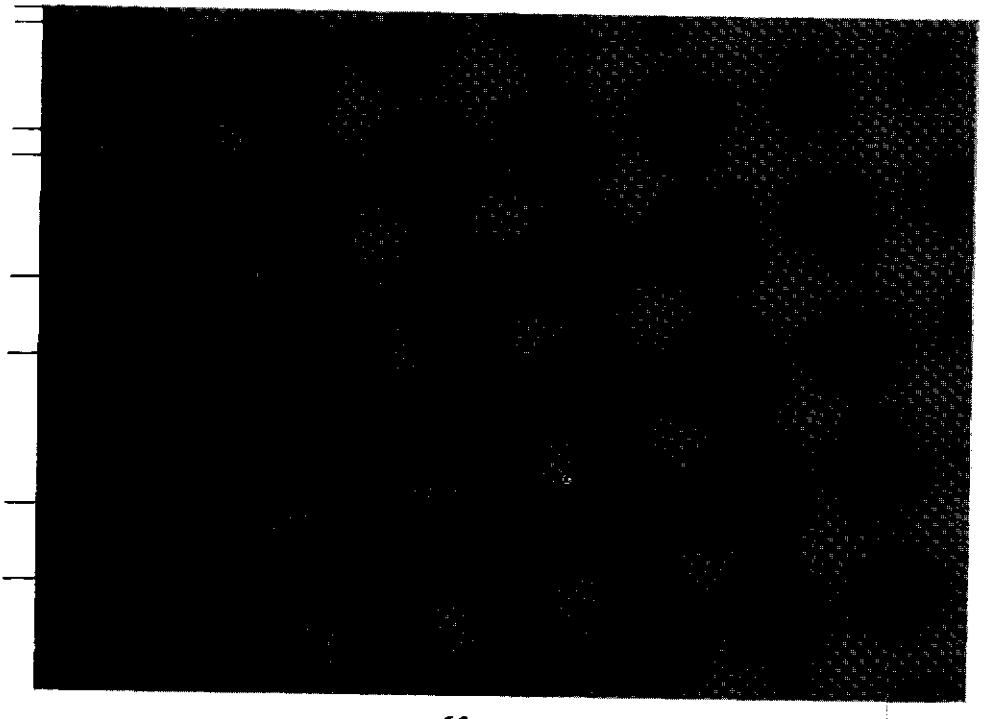

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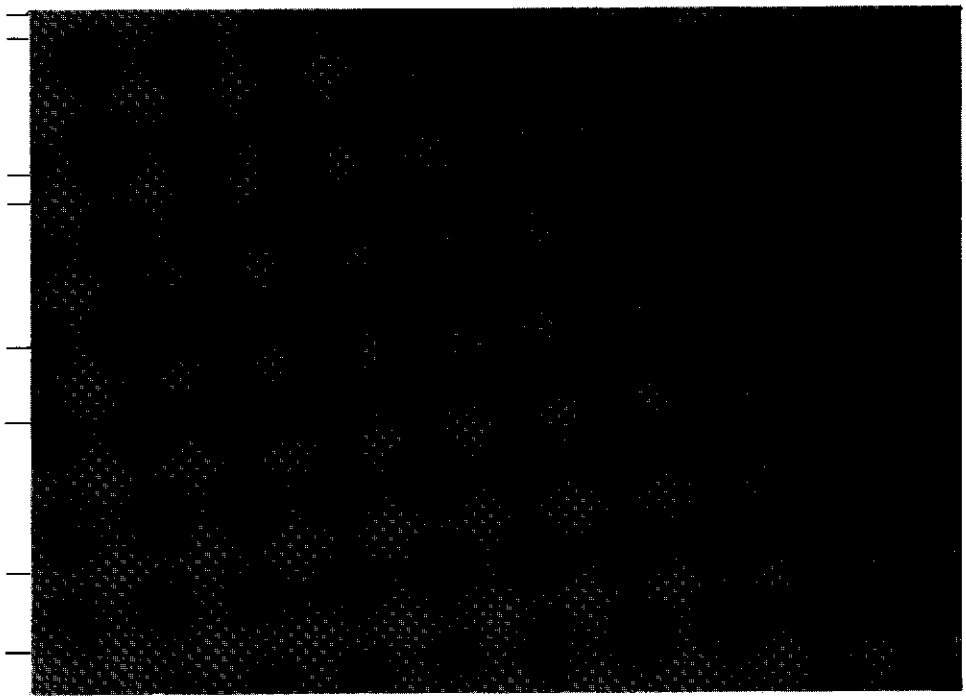

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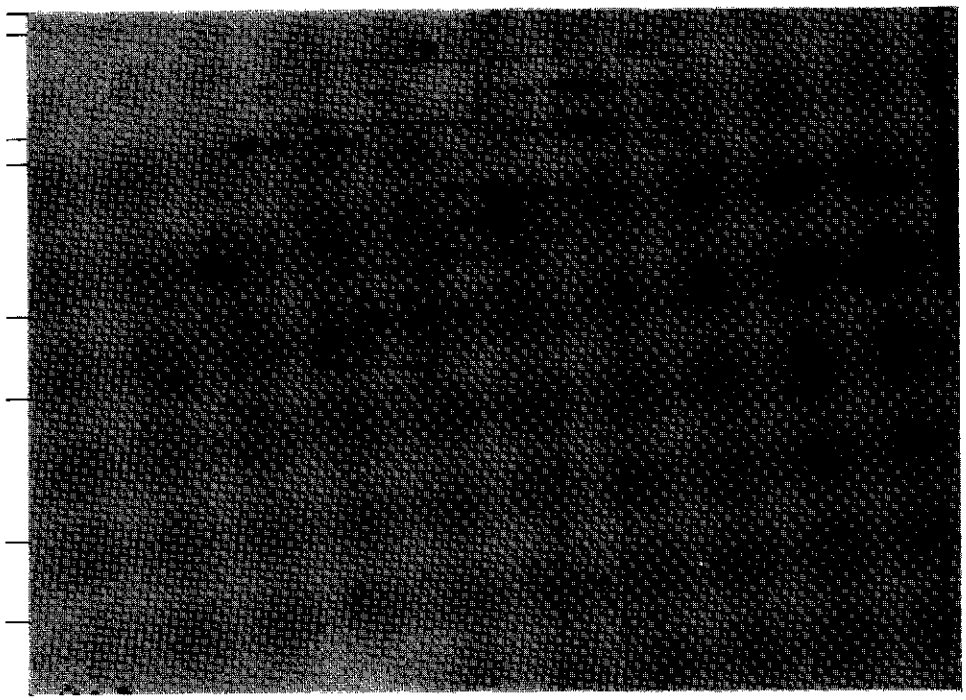

F

Discussion

Although the development of the first leaf of oats does not seem to be characterized by major alterations in the patterns of proteins that are present (Klerk and Van Loon, 1989) or synthesized, specific changes do occur that seem to be related to its functioning. As also noted by other workers (Callow, 1974; Thomas and Stoddart, 1980; Thimann, 1980) the synthesis of Rubisco rapidly declines from leaf emergence to maturity, but the protein is still relatively abundant late in senescence, indicating that it is turning over very slowly. The same trend holds for the other chloroplast-associated proteins, but in contrast to Rubisco some synthesis is still apparent in the late stage of senescence (cf. Brady and Scott, 1977). For many proteins similar relative intensities are found up on labelling and silver staining (Klerk *et al.*, 1989) and these may turn over at a moderate rate. However, various proteins are synthesized at high rates but do not accumulate to high levels, indicating that they must be subject to enhanced turnover. This situation applies to the high-molecular-weight proteins and the proteins marked N in box I. The former are still synthesized at relatively high rates when senescence proceeds, but at this stage they are becoming less intense or barely detectable on silver-stained patterns (Klerk and Van Loon, 1989).

The differences in the synthesis rates of, on the one hand, chloroplast proteins and, on the other hand, the senescence-associated proteins, are sufficiently distinctive to allow the stage of development of the leaf to be defined. The availability of labelled methionine in the distal segment was fairly constant throughout development, but because of the endogenous pool is declining (Van Loon *et al.*, 1987), the specific activity of ^{35}S-methionine is likely to increase substantially. Thus, even though protein synthesis in the older leaves must have declined, sufficient label was incorporated into protein for visualization of the full spectrum of proteins synthesized. Clearly, many proteins are still being synthesized at a late stage of senescence, suggesting that the decrease in protein content is largely due to increased degradation. It has been suggested that senescence is a consequence of the cessation of chloroplast RNA-synthesis (Ness and Woolhouse, 1980; Spiers and Brady, 1981). However, the control of protein degradation seems to be equally important (cf. Thomas and Stoddart, 1980).

The requirement for *de novo* protein synthesis for senescence to proceed

is not easily accommodated within this view. No synthesis of the major proteases appears to be required (Van Loon *et al.*, 1987). Inhibitor studies have shown that leaf senescence is hardly sensitive to the transcription inhibitor actinomycin D (Von Abrams, 1974; Thomas, 1975), but is delayed by inhibitors of translation on particularly cytoplasmic ribosomes, such as cyclohexamide (Martin and Thimann, 1972; Von Abrams, 1974; Thomas, 1975; Makovetzki and Goldsmidth, 1976). Such observations suggest that leaf senescence is regulated by changes in the translation of stable mRNA's synthesized early in development, rather than by changes in transcription. By studying changes in translatable mRNA's during senescence of primary oat leaves by 2D-PAGE, Malik (1987) observed that many mRNA's disappeared, but that two mRNA's, translated into polypeptides of about 40 kDa, appeared at the start of senescence. However, these mRNA's were expressed only in the light and not when plants senesced more quickly in darkness. None of the proteins identified in our study appears to correspond to these translation products.

It seems of interest, however, that some of the high-molecular-weight proteins H, and particularly the three proteins marked N, show strongly enhanced synthesis at the later stages of development, while not accumulating to any sizable amount. The synthesis of these proteins is associated with senescence and if they do indeed function in this process, continued protein synthesis would be required for senescence to proceed. Under these conditions, senescence may depend not so much on the induction of new proteins, but rather on the enhanced synthesis of specific proteins, perhaps through preferential translation from stable mRNA's.

Acknowledgments

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References

Bradford MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye

binding. Anal Biochem 72: 248-254.

Brady CJ, NS Scott 1977 Chloroplast polyribosomes and synthesis of fraction1 protein in the developing wheat leaf. Aust J Plant Physiol 4: 327-335

Callow JA 1974 Ribosomal RNA, Fraction I protein synthesis, and ribulose diphosphate carboxylase activity in developing and senescing leaves of cucumber. New Phytol 73: 13-20

Klerk H, LC Van Loon 1989 Two-dimensional gel electrophoretic changes in soluble proteins during development of primary oat (*Avena sativa* L.) leaves. Chapter 1.

Klerk H, S Tophof LC Van Loon 1989 Metabolism of proteins during development of the first leaf of oats (*Avena Sativa L.*). I *In vivo* labelling of the proteins in the first leaf of intact plants. Chapter 3.

Klerk H, A Jespers 1989 GELSCAN, a personal computer program to compare protein patterns in two-dimensional polyacrylamide gels. Chapter 7.

Makovetzki S, EE Goldsmidth 1976 A requirement for cytoplasmic protein syntheses during chloroplast senescence in aquatic plant *Anacharis canadensis*. Plant Cell Physiol 17: 859-862

Malik NSA 1987 Senescence in oat leaves: Changes in translatable mRNA's Physiol Plant 70: 438-446

Martin C, KV Thimann 1972 The role of protein synthesis in the senescence of leaves. I. The formation of proteases Plant Physiol 49: 173-176

Ness PJ, HW Woolhouse 1980 RNA synthesis in *Phaseolus* chloroplast. II. Ribonucleic acid synthesis in chloroplasts from developing and senescing leaves. J Exp Bot 31:235-245

Spiers J, CJ Brady 1981 A coordinated decline in the synthesis of subunits of ribulose bisphosphate carboxylase in aging wheat leaves. II. Abundance of messenger RNA. Aust J Plant Physiol 8: 603-618

Thimann KV 1980 The senescence of leaves. *In* Thimann KV, Ed, senescence in plants. CRC Press, Boca Raton, FL. pp 85-115

Thimann KV, RR Tetley, T Van Thanh 1974 The metabolism of oat leaves during senescence. II. Senescence in leaves attached to the plant. Plant Physiol 54: 859-862

Thomas H, 1975. Regulation of alanine aminotransferase in leaves of *Lolium temulentum* during senescence. Z Planzenphysiol 74: 208-218

Thomas H, JL Stoddart 1980 Leaf senescence. Ann Rev Plant Physiol 31: 83-111

Van Loon LC, HCPM Van der Valk, AJ Haverkort, GL Lokhorst 1987 Changes in

protease activity in leaves during natural development and acclerated aging upon detachment. J Plant Physiol 127:339-353

Van Telgen HJ, LC Van Loon 1984 Two-dimensional gel electrophoretic analysis of chromatin-associated proteins from leaves of different species and varieties of Nicotiana. J Plant Physiol 118: 285-296

Von Abrams GJ 1974 An effect of ornitine on degradation of chlorophyll and protein in excised leaf tissue. Z Planzenphysiol 72: 410-421

Wittenbach AV 1977 Induced senescence of intact wheat seedlings and its reversibility. Plant Physiol 59: 1039-1042

Woolhouse HW 1978 Senescence processes in the leaf cycle of flowering plants. Bioscience 28: 25-31

Yu SM, CH Kao 1981 Retardation of leaf senescence by inhibitors of RNA and protein synthesis. Physiol Plant 52:207-210

Chapter 5

Metabolism of proteins during development of the first leaf of oats (*Avena sativa* L.).

III Protein degradation *in vivo* and *in vitro*.

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Summary

The primary leaves of 7- and 15-days-old oat plants were labelled with ^{35}S-methionine and the *in vivo* degradation of the synthesized proteins was followed by two-dimensional-polyacrylamide gel electrophoresis. Of the about 300 spots that could be distinguished, a large number turned over quickly (disappearing spots), while a smaller number was degraded very slowly (persisting spots). This pattern of breakdown was compared to that in soluble-protein extracts at pH 5.5 and 7.5., the optimal pH values of the acidic and neutral proteases, respectively. Although 75% of the proteins were degraded similarly *in vivo* and *in vitro*, substantial differences in the relative rates of degradation of the other proteins occurred under the different conditions. Notably, ribulosebisphosphate carboxylase (Rubisco) was degraded very slowly *in vivo*, but rather quickly and in a different way *in vitro* at pH 5.5. The results indicate that compartmentation *in vivo* must largely determine the accessibility of the proteases to their protein substrates. Whereas cytoplasmic proteins could be imported into the vacuole for degradation, chloroplast proteins, such as Rubisco, are likely to be degraded by minor proteases within the organelle.

Introduction

Much research has been conducted on the regulation of the synthesis of proteins during plant development from seed germination to plant maturity,

but comparatively little is known about the factors governing protein turnover and breakdown. In leafy organs, net protein degradation is already apparent at the time when the leaf becomes fully-grown, and is accelerated thereafter (Nooden and Leopold, 1980; Thomas and Stoddart, 1980; Thimann, 1980, Kierk and Van Loon, 1989). Degradation of chloroplast proteins, particularly of ribulosebisphosphate carboxylase / oxigenase (Rubisco) is evident at an early stage of senescence, whereas cytoplasmic proteins appear to be retained for a longer time (Callow, 1974; Thomas and Stoddart, 1980). Finally, when compartmentation is lost , rapid loss of the remaining proteins and withering of the leaf are followed by leaf death (Woolhouse and Jenkins, 1983). The apparent gradual increase in protein degradation during leaf development could be the result of 1) increased protease activity due to *de novo* synthesis or enzyme activation, 2) decompartmentation or redistribution of protease, enabling the enzyme to come into contact with its protein substrates, 3) characteristics of proteins favoring susceptibility to degradation, and 4) increased transport of proteins into protease-containing compartments.

In detached oat leaves, senescing while floating in the dark, Martin and Thimann (1972) observed increases in the activities of the two major proteases, an acidic and a neutral one, with pH optima of 4.5 and 7.5, respectively. However, depending on the conditions of incubation of the leaf segments, protein breakdown occurred at a similar rate, whether the protease activities increased or not (Van Loon *et al.*, 1987). Similarly, during the natural senescence of leaves on intact plants no increases in the protease activities were observed before more than 70% of the protein had been lost. Although protease activity may increase with age (Van der Valk and Van Loon, 1988), young leaf cells already possess sufficient protease activity to degrade all proteins present (Van Loon *et al.*, 1987). It was therefore proposed that the proteases are spatially separated from their protein substrates. During senescence, the protease-containing compartment(s) might become leaky, leading to contact between the enzyme and its substrates and, consequently, accelerated loss of protein. In a test of this hypothesis, Van der Valk and Van Loon (1988) demonstrated that the acidic protease is located in the vacuole and remains compartmentalized there throughout the period in which leaf protein content decreased by more than 50%. The neutral protease was not present in the vacuole but its subcellular localization could not be further established. However, in view of its rather restricted activity (Van Loon *et al.*, 1987),

it was considered unlikely to serve a general role in protein breakdown.

The vacuolar protease could still function in the degradation of cytosolic proteins if these were transferred into the vacuole. In that case the rate of protein breakdown would depend on a more or less specific transport of protein substrates across the tonoplast. Cytoplasmic proteins have been shown to be present in vacuoles (Canut *et al.*, 1985, 1986) but the mechanism for their import has not been established, nor is it clear to what extent such a mechanism could be responsible for general protein degradation. There is evidence that minor endo- and exoproteases are present outside the vacuole which might also participate in protein breakdown during senescence. Some reports indicate that chloroplasts (Waters*et al.*, 1982; Martinoia *et al.*, 1983; Thayer *et al.*, 1988) and mitochondria (Chua and Smith, 1979) contain minor proteases that might function in the degradation of organellar proteins.

Alternatively, the properties of the proteins themselves might influence their rate of degradation. Drawing on studies on protein breakdown in mammals, bacteria, and yeasts , Davies (1982) reviewed the hypothesis that a high molecular weight and a low isoelectric point would predispose a protein to rapid degradation, whereas glycosylation would have a stabilizing influence. However, in surveys of the rates of protein degradation in barley (Hordeum vulgare cv. Proctor) leaves (Coates and Davies, 1983) and *Lemna* fronds (Ferreira and Davies, 1987 a,b), no such relationship was apparent.

In the present study, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) was used to follow the loss of label from newly synthesized proteins in the first leaf of oats *in vivo*. The resulting pattern was compared with the patterns of degradation in leaf extracts incubated at the pH optima for the acidic and neutral protease activities. In this way, it was possible to estimate whether protein degradation *in vivo* reflects the specificity of the degradation by any of these proteases *in vitro*.

Material and methods

Plant material.

For studying protein breakdown *in vivo*, oats (*Avena sativa* L. cv. Victory) was grown hydroponically in a growth cabinet, as described

previously (Klerk *et al.*, 1989a). At regular intervals between 7 and 37 days after sowing both sides of the 4.5 cm distal part of the primary leaf of each plant were gently rubbed three times with ethanol-saturated Kleenex tissue paper and immersed for 24 hours in 1.4 ml of a solution containing 450 kBq ^{35}S-methionine (44 TBq/mmol). After labelling the plants were further grown for up to 14 days under the same conditions, during which the treated segments were harvested at regular intervals. The leaves were not chased with non-labelled methionine because large amounts of methionine may influence leaf senescence through increased ethylene production (L.C. Van Loon, unpublished result).

Plants grown in a greenhouse (Van Loon *et al.*, 1987) were used for preparing extracts to study protein breakdown *in vitro*. Seven-day-old plants were used because both the acidic and neutral protease activities are high at this stage (Van Loon *et al.*, 1987). Similar 4.5 cm distal parts of the primary leaves were harvested.

Determination of protein breakdown in vivo.

Leaf segments labelled with ^{35}S-methionine were extracted individually with 1.5 ml extraction buffer (0.1 M Tris-HCl, 10 mM EDTA, 10 mM 2-mercaptoethanol, 0.1 M NaCl and 0.1% ascorbic acid, pH 7.7) by grinding in a mortar with a pestle with some sea sand and 5% (w/w) polyvinylpyrrolidone at 2 ^{0}C. The homogenate was centrifuged for 10 min in an Eppendorf centrifuge. The supernatant was transferred to a clean Eppendorf tube and centrifuged again for 15 min. A 10 µl sample of the supernatant was used for determining the amount of ^{35}S-methionine present in the soluble fraction by means of liquid scintillation spectrometry. The amount incorporated into protein was determined after TCA precipitation, as described previously (Klerk *et al.*, 1989a,b).

The proteins present in the supernatant were further purified by phenol extraction and acetone precipitation, as described previously (Klerk and Van Loon, 1989). The protein precipitate was washed twice with 80% and 100% acetone, respectively, dried under vacuum, and stored at -20^{0}C before analysis by 2D-PAGE.

Determination of protein breakdown in vitro.

Leaf segments from non-labelled plants were harvested and a total of 6 g was extracted with 30 ml extraction buffer and centrifuged as described by Klerk and Van Loon (1989). The supernatant was directly used without the phenol extraction.

Since endogenous proteins are readily degraded at pH 5.5 but at a lower rate at lower pH values (Van Loon *et al.*, 1987), hydrolysis by the acidic protease was followed at pH 5.5 and by the neutral protease at pH 7.5. The extracts were mixed with an equal volume of either phosphate-citrate buffer, pH 5.5, or 0.2 M Tris-HCl buffer, pH 7.5, and incubated at 40 °C. At regular intervals 1 ml samples were withdrawn. In one of the samples proteins were precipitated by adding 250 µl of 25% TCA. After centrifugation the precipitate was dissolved in 1 ml 0.1 N NaOH for protein determination. Proteins in other samples were phenol-extracted for 2D-PAGE as described above.

Two-dimensional gel electrophoretic analysis

2D-PAGE was performed as described in detail previously (Klerk and Van Loon, 1989). In each case, 20 µg of protein was applied to the gel. Thus, patterns for the later stages of leaf development, in which protein content is lower, quantitatively overestimate the presence of individual proteins (cf. Klerk and Van Loon, 1989; Klerk *et al.*, 1989b). In the first dimension, IEF extended from pH 5 to 8, and a 12.5 % polyacrylamide SDS slab gel was used in the second dimension. Gels were stained with silver as described by Van Telgen and Van Loon (1984). Fluorography was performed using preflashed Kodak X-Omat film to allow a quantitative estimation of the amount of label retained (Klerk *et al.*, 1989b), and a qualitative comparison of the protein spots was made with the computer program GELSCAN, as described by Klerk and Jespers (1989).

Determination of protein content

Soluble protein was determined using the method of Bradford (1976). BSA dissolved in the same buffer as the proteins to be determined was used as a standard.

Results

Fate of in vivo *labelled proteins*

Plants were labelled at 7 and 15 days after sowing. These times correspond to stages where the first leaf is rapidly growing, and is entering the stage of senescence, respectively. Protein content in the distal part is still increasing at 7 days, whereas at 15 days about 20% of the maximum protein content reached around day 9 has been lost (Klerk *et al.*, 1989b). These stages thus represent situations in which protein metabolism is dominated by synthesis and degradation, respectively. Table 1

Table 1. Incorporation of ^{35}S-methionine into 4.5 cm distal segments of the first leaf of 7 and 15 days-old oat plants after labelling for 24 h

Amount of label per segment	Plant age	
	7 day old	15 day old
Supplied in labelling solution	2.7×10^8 dpm	2.7×10^8 dpm
Taken up and retained	8.0×10^6 dpm	6.0×10^6 dpm
TCA-precipitable	3.0×10^6 dpm	2.2×10^6 dpm
Protein specific activity (dpm/µg)	3.0×10^3 dpm	3.5×10^3 dpm

shows the incorporation of ^{35}S-methionine in the leaf segments at the two times. In accordance with previous observations (Klerk *et al.*, 1989b), a higher specific activity for the protein was attained at 15 than at 7 days probably because of a smaller aminoacid pool.

During the subsequent 14 days no more than 35 and 60 % of the label was lost from the leaf apices labelled at 7 and 15 days, respectively, as a result of redistribution among the other plant parts (Fig. 1A). Although unincorporated methionine is quickly metabolized within the leaf (cf. De Laat and Van Loon, 1981), the possibility cannot be excluded that some labelled methionine liberated through protein degradation was reused for protein synthesis.

The fate of the labelled proteins was followed over the 14 days following the uptake of ^{35}S-methionine. From 7 to 21 days, the leaf passes through the stage of maturity into senescence, whereas from 15 to 29 days

senescence proceeds and almost all protein is lost from the leaf. The

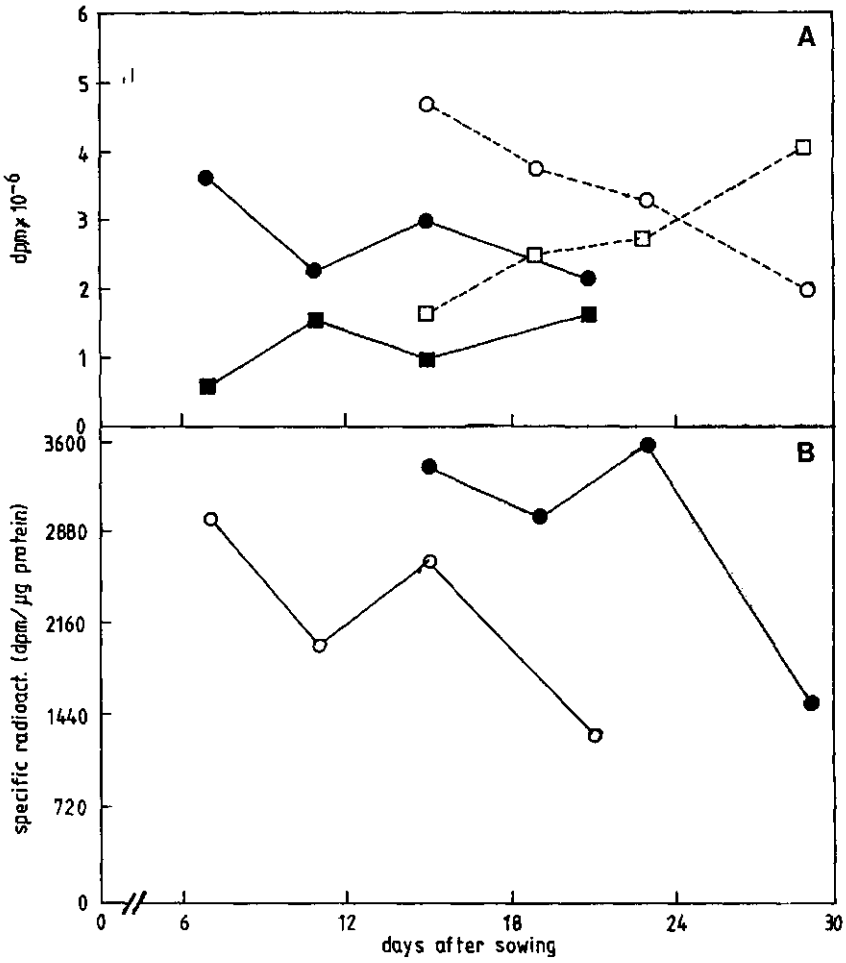

Figure 1. A: Changes in the distribution of ^{35}S after a 24-h labelling of the distal 4.5 cm segment of the first leaf on (●,■) 7- and (○,□) 15 days old plants with Bq ^{35}S-methionine: (○,●) distal 4.5 cm of the primary leaf; (□,■) rest of the plant. B: Changes in the specific radioactivity of the soluble protein in the distal 4.5 cm of the first leaf of (○) 7- and (●) 15-days-old plants. after a 24-h labelling with ^{35}S-methionine.

course of the specific activities of the labelled protein is depicted in Fig. 1B. Although the specific activities tended to decline in the first days after labelling, suggesting loss of label from proteins that are rapidly turning over, it remained high until at least 8 days after

labelling. During this period protein content decreased at an almost linear rate (cf. Van Loon *et al.*, 1987). Hence, the remaining labelled proteins seemed to be fairly stable. Only afterwards, during the second week after labelling, the specific activity of the protein decreased more rapidly, indicative of increased turnover. This pattern held for plants labelled either at 7 days or at 15 days, reaching 1.4×10^3 and 1.5×10^3 dpm/µg protein, respectively, after 14 days of turnover.

These conclusions were supported when the fate of the individual proteins by 2D-PAGE analysis was followed (Figs 2A, B). The pattern shown in Fig. 2A represents the proteins immediately after synthesis at day 8 (t=0), and corresponds to the pattern of proteins synthesized in expanding leaves, as described previously (Klerk *et al.*, 1989b), A total of 306 spots could be distinguished and a series of high-molecular-weight proteins (marked H), known to be synthesized by the end of leaf expansion (Klerk and Van Loon, 1989b), were clearly labelled. furthermore a series of proteins could be distinguished of about the same molecular weight, previously identified as chloroplast-associated proteins (Klerk and van Loon, 1989) and therefore marked C. The fate of the spots are indicated by white (dissappearing spots) and black (persistent spots). At day 11 (t=4) the number of labelled spots had decreased to 270 and the intensity of a great many spots all over the gel, particularly in the low-molecular-weight region, was strongly reduced (e.g., spots in box I). Both the high-molecular-weight proteins H and C were little affected. Relatively few changes occurred during the following days. At day 15 (t=8) declining spots had further decreased in intensity or disappeared, whereas the high-molecular-weight proteins were still prominent, leaving 180 spots. By day 21 (t=14) virtually all declining spots, as well as the chloroplast-associated proteins, had disappeared and the intensity of the about 100 remaining

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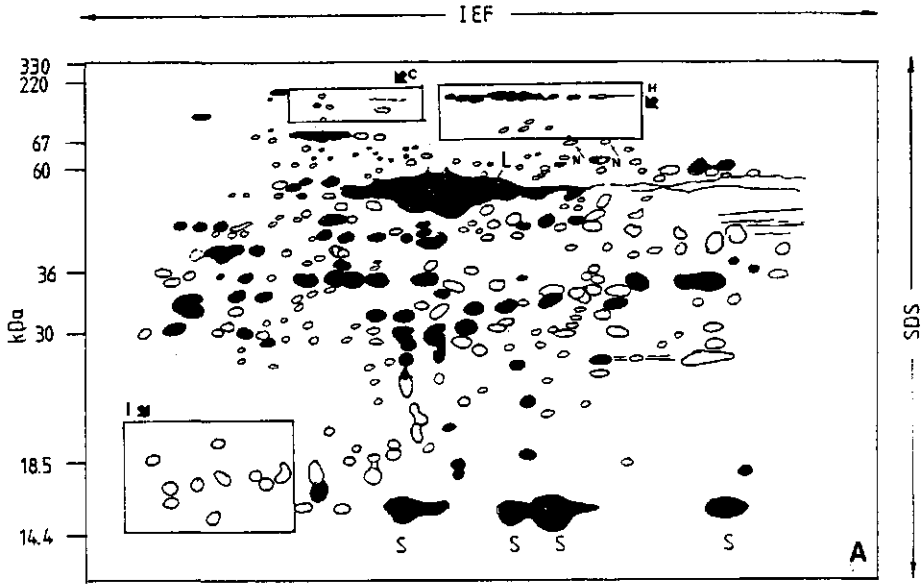
Figure 2. *In vivo* degradation of soluble proteins in the distal 4.5 cm of the first leaf after labelling 7- plants (A) and 15-days-old plants (B) for 24 h with ^{35}S-methionine. A composite map of the locations and fate of protein spots based on a series of fluorograms of 2D-PAGE patterns 0, 4, 8, and 14 days after the end of the labelling period. White spots are disappearing spots and black spots are persistent spots. The positions of the molecular-weight markers thyroglobulin (330 kDa), ferritin (half unit) (220 kDa), BSA (67 kDa), catalase (60 kDa), lactate dehydrogenase (36 kDa), carbonic anhydrase (30 kDa), ferritin (18.5 kDa), α-lactalbumin (14.4 kDa) are indicated. C= chloroplast-associated polypeptedes; D=disappearing polypeptides; H= high molecular-weight polypeptides; L=Rubisco large subunits; N= senescence-associated polypeptides; S= Rubisco small subunits. Spots of particular interest are boxed (see text).


IEF
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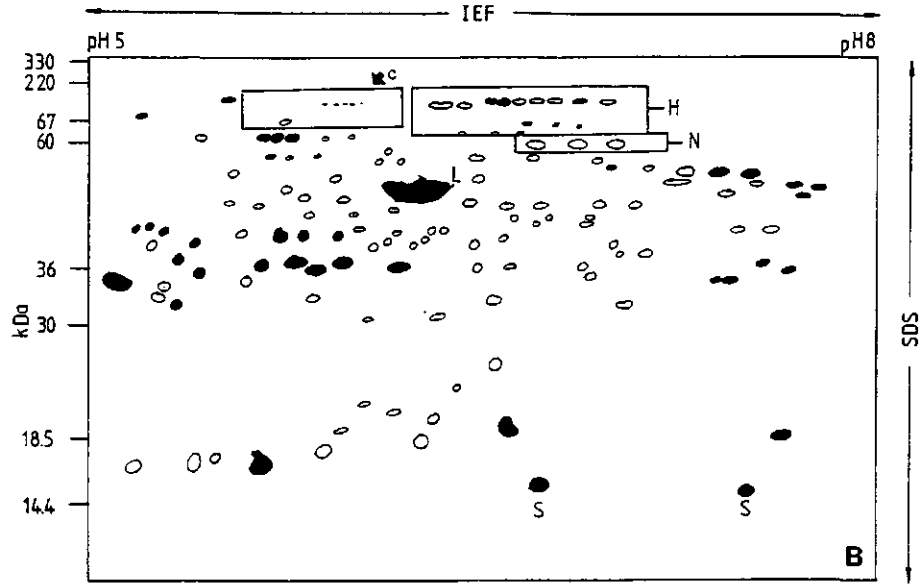
IEF
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spots was also decreasing. At this stage, both the large and small subunits of Rubisco still formed strongly labelled spots, indicating that Rubisco is turning over very slowly *in vivo*. Whereas the H proteins were among the most stable at earlier times, they were now also being degraded. Of the remaining proteins, no special characteristics were evident. Certainly it did not appear that a particular range of molecular weight or iso-electric point predisposed proteins to rapid degradation. Thus, starting from 7-days-old plants the pattern of protein breakdown *in vivo* seemed to comprise a rather quick turnover of a large number of proteins and a slow decline of a smaller number of persisting spots.

As compared to Fig. 2A the pattern depicted in Fig. 2B reflects the change in the proteins synthesized in leaves during incipient senescence, 15 days after sowing. At this stage Rubisco was hardly synthesized any more (cf. Klerk *et al.*, 1989b) and the chloroplast-associated proteins marked C were virtually unlabelled. A total of 158 spots was distinguishable. Among these the H proteins were relatively prominent and the three proteins that are synthesized during senescence, marked N, were also clearly labelled. At day 19 (t=4) several previously identified disappearing spots (White spots) were strongly reduced in intensity or were already gone, leaving about 132 spots. Whereas the H proteins were quite resistant to degradation the N proteins were no longer discernible at this stage. Most of the remaining spots were persistent (Black spots), as evident after 8 or 14 days. At day 29 (t=14) the pattern, of about 80 spots, differed from that shown by the black spots in Fig. 2A only by the absence of those proteins that were not synthesized at day 15. Thus, also during leaf senescence there is a fast turnover of a large number of proteins and a slow decrease of a small number of persistent spots.

In vitro *protein breakdown*

The course of breakdown of total soluble proteins in extracts is shown in Fig. 3. *In vitro* protein degradation occurred at a much faster rate than *in vivo*. Within 4 h 70% of the protein had been degraded at pH 5.5 and 40% at pH 7.5. Thereafter, protein breakdown slowed down, and 20 and 60% were still detectable after 21 h of incubation at pH 5.5 and 7.5, respectively. The lack of further degradation at pH 7.5 is probably due to the instability of the neutral protease (Van der Valk and Van Loon, 1988).

The breakdown of the proteins, as followed by 2D-PAGE and silver staining, is shown in Figs 4A and B. Fig. 4A shows the pattern obtained by

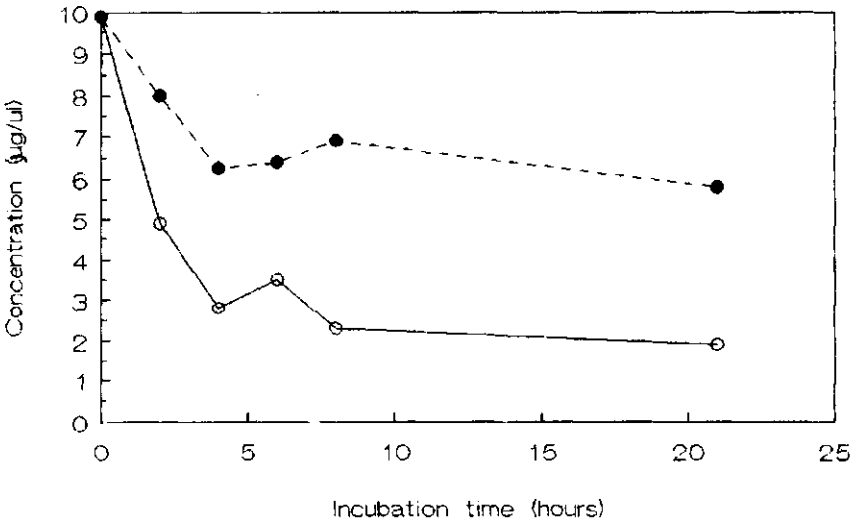

Figure 3. Loss of protein during incubation of extract from the distal 4.5 cm of the first leaf of 7-days-old plants *in vitro* at 40 °C and (O) pH 5.6 and (●) pH 7.6.

bringing the extract at pH 5.6 before the start of incubation (t=0). Disappearing spots are white, persisting spots are black and new spots or regions with degradation products are gray. This pattern is comparable to that of 7-days-old leaves described previously (Klerk and Van Loon, 1989) and corresponds to the labelling pattern shown in Fig. 2A. Both subunits of Rubisco (L and S) and the high-molecular-weight proteins H, as well as the chloroplast-associated proteins C are marked. The overall pattern is comparable with Fig. 2A, except that it also includes proteins that are present but not synthesized at this stage. During a 2-h incubation at pH 5.6 the pattern remained similarly complex. However, some spots were disappearing rapidly. Other spots seemed to shift positions (See arrows). Thus, the row of spots encompassing the L subunit of Rubisco shifted to more basic pI's. Higher pI's were associated with slightly lower molecular weights. Similar shifts were observed for the high-molecular-weight spots marked H. In this case additional spots with higher pI's became apparent. In contrast in box I two proteins that appeared strongly resistant to proteolysis seemed to give rise to spots with lower pI's that were, in

turn, more susceptible to degradation. After 4 h of incubation most of the weak spots were no longer discernible. Both Rubisco and the high-molecular-weight proteins H had largely disappeared. At t=21 h only remnants of these proteins were present but a small number of proteins had been little affected. In no way did this pattern resemble that of the proteins persisting during natural leaf development (cf. Fig. 2A).

During incubation at pH 7.5 (Fig. 4B) the pattern of protein breakdown was substantially different. After 2 h of incubation the complexity of the pattern was clearly diminished. Except for the shift in pI of the two spots in box 1, no changes in the positions of other spots were observed. After 4 h a further decrease in complexity was observed, largely because minor spots had disappeared, and persistent spots were clearly evident. Although protein concentration did not decrease to any significant extent between 4 and 21 h of incubation (Fig. 3), Rubisco did seem to be further degraded, probably to lower-molecular-weight peptides, and the high-molecular-weight proteins were no longer discernible. An increase in background staining suggests that polypeptides might have been further modified by peptidase action.

Comparative analysis of Figs 2 and 4 indicates that of the most prominent spots, 120 spots were degraded *in vivo* and / or *in vitro*. Of these, 89 disappeared similarly under all conditions, but the fate of 31 differed *in vivo* and *in vitro* (Table 2). Some disappearing *in vivo* did not disappear *in*

Table 2. Patterns of protein degradation of 120 prominent spots[a]

Fate *in vivo*[b]	Fate *in vitro*[b]		Number of spots
	pH 5.5	pH7.5	
D	D	D	89
D	P	D	2
D	D	P	3
D	P	P	9
P	P	D	4
P	D	P	3
P	D	D	10

[a]Spots were present in Figs 2 and 4.
[b]D=Disappearing, P=Persisting.

vitro, and some that were degraded *in vitro* were retained *in vivo*. Thus, although the majority of the proteins was degraded under all conditions, proteolysis in extracts differed from protein breakdown *in vivo*. *In vitro* at pH 5.5 Rubisco was degraded very differently from the *in vivo* situation,

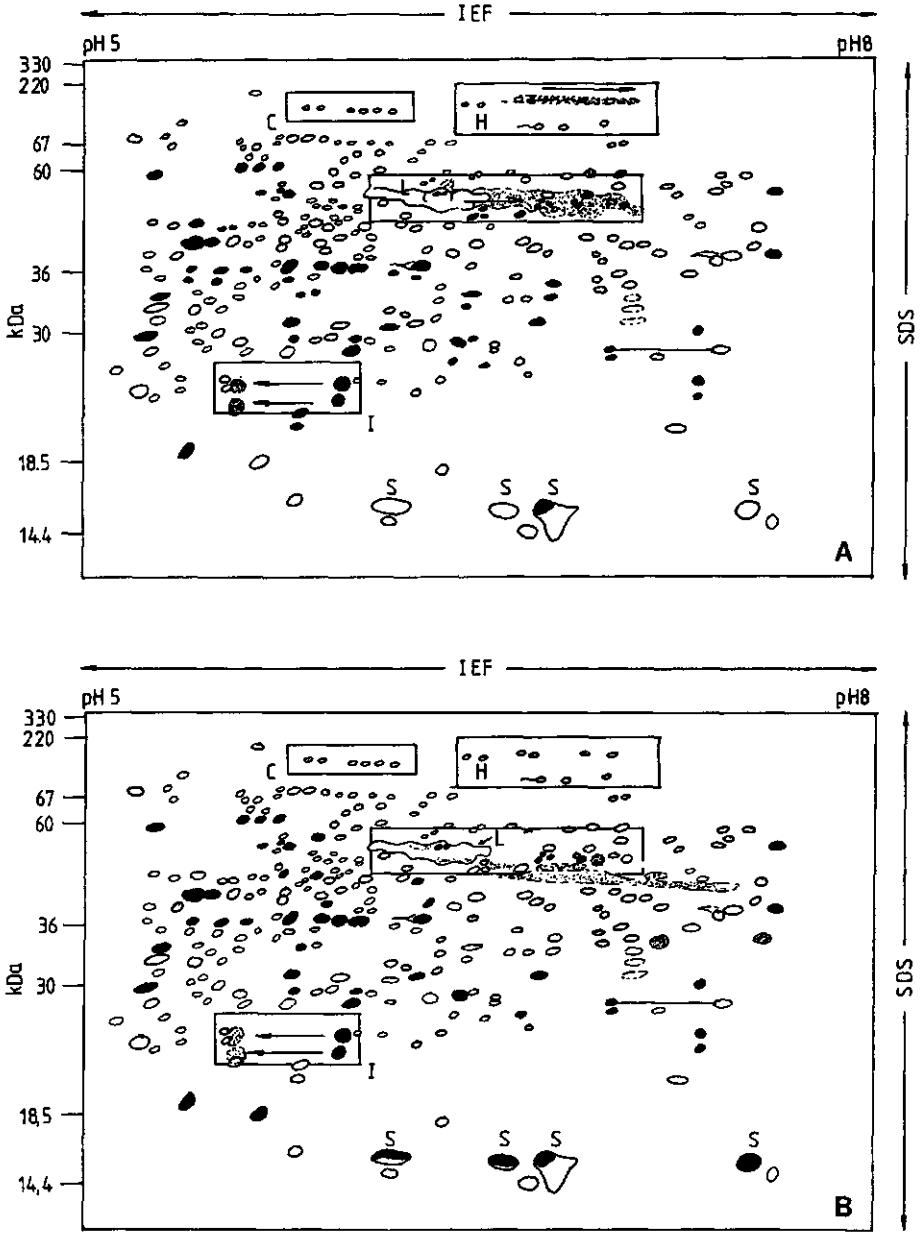

Figure 4. *In vitro* degradation of soluble proteins from the distal 4.5 cm of the first leaf of 7-days old plants. A composite maps based on silverstained 2D-PAGE patterns after incubation at pH 5.5 (A) or pH 7.5 (B) for 0, 2, 4 and 21 h. Gray regions or spots are new spots and/or degradation products. Arrows indicate shifts in the protein pattern. Other markings as in Fig 3.

whereas at pH 7.5 the pattern of *in vivo* and *in vitro* degradation were less dissimilar. Thus proteolysis *in vitro* at pH 7.5 appears to resemble protein breakdown *in vivo* more than exposing extracts to pH 5.5.

Discussion

Independent of the stage of development at which proteins were labelled, a relatively rapid turnover of a rather large number of disappearing proteins occurred, followed by a relatively slow decline of a smaller number of persisting proteins. Even 14 days after labelling, a considerable number of labelled spots was still conspicuous, indicating that in primary oat leaves many proteins may turn over with half-lives exceeding 1-2 weeks. Interestingly senescence-associated proteins N, that are synthesized abundantly in the later stages of leaf development (Klerk and Van Loon, 1989b) turned over rapidly, in accordance with previous observations that they do not accumulate to any significant extent. The patterns of disappearing and persisting spots were quite similar early in development and during the stage of senescence. This common pattern strongly suggests that the same mechanism(s) of protein turnover are operating at these different stages, and argue against a new mechanism of proteolysis becoming active at the onset of senescence.

Several studies on animal, bacterial and yeast proteins suggested that *in vivo* half-lives of proteins are determined by their physical properties rather than by the specificity of the proteolytic system (Goldberg and St. John 1974, 1976). Most of the evidence supporting this general hypothesis has been obtained from correlations between *in vivo* rates of degradation, determined by the double-isotope method, and the physical properties of the proteins such as charge and size, as evidenced by SDS gel electrophoresis and isoelectric focusing. Preferential degradation occurred in proteins with high molecular weights and low pI's. However, no such correlation was evident in our study on oat leaf proteins. Similar findings have been reported for barley and *Lemna* (Coates and Davies, 1983; Ferreira and Davies, 1987a, b). It was suggested by Ferreira and Davies (1987a) that the good correlation found by Dice *et al.* (1973) for pea epicotyls, might have been artificial. More recently other physical properties of proteins have been suggested to be responsible for rapid turnover. Bachmair *et al.* (1986) found that the N-terminal amino acid of a protein is a determinant of its

half-life *in vivo*. Moreover, Rogers *et al.* (1986) observed that proteins with intracellular half-lives of less than 2 h contain one or more regions characterized by the sequence proline, glutamic acid, serine and threonine (PEST).

Since protease activity does not increase during oat leaf development (Van Loon *et al.*, 1987), the major protease remains compartmentalized in the vacuole up to a late stage of senescence (Van der Valk and Van Loon, 1988), and special characteristics of disappearing and persistent protein spots were not evident, transport of proteins into protease-containing compartments might be considered as a mechanism regulating protein breakdown. According to Canut *et al.* (1985,1986), proteins might be tagged to be degraded in the vacuole, which in plant cells functions as a lysosome (Matile, 1978) and contains the acidic protease (Van der Valk and Van Loon, 1988). Although 89 of the 120 most prominent protein spots identified were degraded similarly *in vivo* and *in vitro*, particularly at pH 5.5, considerable differences were noticeable, indicating that only a selective transport of proteins into the vacuole might well explain the results described. Such a mechanism might be operative for cytoplasmic proteins, whereas chloroplastic proteins, such as Rubisco, and perhaps proteins of other organelles would be degraded *in situ*, probably by minor proteases (cf. Thayer *et al.*, 1988). The fact that, in general, proteins are degraded far more slowly *in vivo* than *in vitro* also indicates that compartmentation *in vivo* determines the accessibility of the proteases to their protein substrates. and , hence, the measure of protein degradation.

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References

Bachmair A, D Finley and A Varshavsky 1986 *In vivo* half-life of a protein is a function of its amino acid residue. Science 234: 179-186

Bradford MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254.

Chua NH GJ Schmidt 1979 Transport of proteins into mitochondria and chloroplasts. J Cell Biol 81:461-483

Callow JA, 1974 Ribosomal RNA, Fraction I protein synthesis, and ribulose diphosphate carboxilase activity in developing and senescing leaves of cucumber. New Phytol 73: 13-20

Canut H, G Alibert, AM Boudet 1985 Hydrolysis of intracellular proteins in vacuoles isolated from Acer pseudoplatanus L. cells. Plant Physiol 81: 460-463

Canut H, G Alibert, A Carrasco AM Boudet 1986 Rapid degradation of abnormal proteins in vacuoles from Acer pseudoplatanus L. cells. Plant Physiol 81:460-463

Coates JB, DD Davies 1983 The molecular basis of the selectivity of protein degradation in stressed senescent barley (*Hordeum vulgare* cv. Proctor leaves. Planta 158: 550-559

Davies DD 1982 Physiological aspects of protein turnover. In D Boulder, B Pathier, Eds, Nucleic acids and proteins in plants I. Encyclopedia of plant Physiology New Series Vol 14A. Springer- Verlag Berlin pp 189-228

De Laat AMM, LC Van Loon 1981 Complications in interpreting precursor/product relationships by labelling experiments. Methionine as the precursor of ethylene in tobacco leaves. Z Plantzenphysiol 103:199-205

Dice JF, PJ Dehlinger, RT Schimke 1973 Studies on the correlation between size and relative degradation rate of soluble proteins. J Biol Chem 248:4220-4228

Ferreira RB, DD Davies 1987a Protein degradation in *Lemna* with particular reference to Ribulose Bisphosphate Carboxylase. I The effect of light and dark. Plant Physiol 83:867-877

Ferreira RB, DD Davies 1987b Protein degradation in *Lemna* with particular reference to Ribulose Bisphosphate Carboxylase. II The effect of nutrient starvation. Plant Physiol 83:878-883

Goldberg AL, AC St John 1974 Intracellular protein degradation in mammals and bacterial gels: part1. Ann Rev Biochem 43: 835-869

Goldberg AL, AC St John 1976 Intracellular protein degradation in mammals and bacterial gels: part2. Ann Rev Biochem 45: 747-803

Klerk H, A Jespers 1989 GELSCAN, a personal computer program to compare

protein patterns in two-dimensional polyacylamide gels. Chapter 7.

Klerk H, LC Van Loon 1989 Two-dimensional gel electrophoretic changes in soluble proteins during development of primary oat (*Avena sativa L.*) leaves. Chapter 2.

Klerk H, S Tophof, LC Van Loon LC 1989a Metabolism of proteins in developing leaves of oats (*Avena sativa* L.). I *In vivo* labelling of the proteins in the first leaf of intact plants. Chapter 3.

Klerk H, S Tophof, LC Van Loon 1989b Metabolism of proteins in developing leaves of oats (*Avena sativa* L.). II Protein synthesis in the first leaf of intact plants. Chapter 4.

Matile P 1978 Biochemistry and function of vacuoles. Ann Rev Plant Physiol 29:193-213

Martin C KV Thimann 1972 The role of protein synthesis in senescence of leaves. I The formation of protease. Plant Physiology 49: 64-71

Martinoia E, U Heck, MJ Dalling, P Matile 1983 Changes in chloroplast number and chloroplast constituents in senescing barley leaves. Biochem. Physiol Planzen. 178: 147-155

Nooden LD, AC Leopold 1980 Phytohormones and the regulation of senescence and abscission. *In* Letham DS, PB Goodwin, TJV Higgins, Eds, Phytohormones and related compounds: A Comprehensive Treatise, ed . Vol 2 Amsterdam Elsevier. pp 329-370.

Rogers S, R Wells, M Rechsteiner 1986 Amino acid sequences common to rapidly degraded proteins: The PEST hypothesis. Science 234;364-368

Thayer SS, HT Choe, S Rausser, RC Huffaker 1988 Characterization and subcelluiar localization of amino peptidases in senescing barley leaves. Plant Physiol 87: 894-897

Thimann KV 1980 The senescence of leaves. In Senescence of plants, KV Thimann Ed. CRC press, Boca Raton, FL. pp 85-115

Thomas H, JL Stoddart 1980 Leaf senescence. Ann Rev Plant Physiol 31: 83-111

Van der Valk HCPM, LC Van Loon 1988 Subcellular localization of protease in developing leaves of oats (*Avena sativa* L.). Plant Physiol 87: 536-543

Van Telgen HJ, LC Van Loon 1984 Two-dimensional gel electrophoretic analysis of chromatin-associated proteins from leaves of different species and varieties of Nicotiana. J Plant Physiol 118: 285-296

Van Loon LC, HCPM Van der Valk, AJ Haverkort, and GL Lokhorst 1987 Changes in protease activity in leaves during natural development and accelerated aging upon detachment. J Plant Physiol 127: 339-353

Waters SP, ER Noble MJ Dalling 1982 Intracellular localization of peptide hydrolases in wheat (*Triticum aestivum* L.) leaves. Plant Physiol 68: 575–579

Woolhouse HW, GI Jenkins 1983 Physiological responses, metabolic changes and regulation during leaf senescence. *In* Dale JE, FY Milthorp, Eds, The growth and function of leaves. Cambridge Univ Press. pp 451–487

Chapter 6

Effects of light and regulators on senescence-related changes in soluble proteins in detached oat (*Avena sativa* L.) leaves.

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Summary

The rate of senescence and the protein patterns of detached oat leaves senescing in either darkness or light were analyzed, and compared to those of leaves in which senescence was delayed by application of the cytokinin benzyladenine or enhanced through the action of abscisic acid.

Senescence of detached leaves in light did not differ significantly from senescence in attached leaves. In darkness, protein was lost at a higher rate, but several proteins showed relative increases. Notably proteins previously characterized as high-molecular-weight proteins and senescence-associated proteins (Klerk *et al.*, 1989b) increased. Changes during incubation in light and darkness were not related to senescence. Cytokinins delayed and abscisic acid accelerated the changes in protein pattern compared to water. Beside changes previously identified in leaves senescing on the plant, detached leaves show alterations that reflect their condition of incubation rather than their developmental progress.

Introduction

Leaf senescence is a developmental process, the rate of which is influenced by both external and internal factors (Nooden and Leopold, 1980; Thomas and Stoddart, 1980; Woolhouse and Jenkins, 1983;). The removal of a leaf from the plant predisposes it to accelerated ageing and this method is often used to study the regulation of leaf senescence (cf. Thimann, 1980). Thus, upon detachment primary oat leaves can be manipulated to senesce prematurely. Notably senescence is accelerated moderately when the leaves are kept in the light, but strongly in darkness (Udvardy *et al.*, 1967;

Shibaoka and Thimann, 1970; Thimann *et al.*, 1974; Van Loon *et al.*, 1987; Malik, 1987). Cytokinins, particularly the artificial cytokinins benzyladenine and kinetin, rather than the natural ones, isopentyladenine and zeatin, are effective in delaying senescence (Varga and Bruinsma, 1973), whereas abscisic acid enhances senescence, particularly in light (Satler and Thimann, 1979; Thimann, 1980). Although ethylene is supposed to be the hormone ultimately responsible for leaf senescence, detached leaves in darkness are so sensitive to this regulator that its stimulation or inhibition only marginally affect the rate at which senescence proceeds (Gepstein and Thimann, 1981). This sensitivity to ethylene appears to be due to a high content of ABA in darkness. This can explain the senescence-promoting effect of ABA in the light. Thus, the rate of senescence of detached oat leaves seems to be determined mainly by the balance of senescing-promoting ABA and senescence-delaying cytokinin.

It is questionable, however, whether the regulation of leaf senescence in detached leaves is similar to that in leaves attached to the plant. Detached leaves are cut off from the supply of nutrients and hormones from other plant parts and cannot export products of the hydrolysis of various types of macromolecules to young growing parts or storage organs. Moreover, detachment engenders a wounding response which may have both enhancing and inhibitory effects on leaf senescence (Giridhar and Thimann, 1985; Thomson *et al.*, 1987). In detached barley leaves Miller and Huffaker (1985) observed the appearance of four endoproteases that did not occur during the senescence of leaves attached to the plant. During the senescence of detached oat leaves Giridhar and Thimann (1985) also reported that extant proteins were retained and new proteins appeared as compared to the changes occurring in attached leaves.

It was shown previously (Van Loon *et al.*, 1987) that the activities of the major proteases in the first leaf of oats change in greatly differing ways during natural senescence and accelerated aging and that similar decreases in protein content occurred whether activities increased or not. Thus, it became of interest to investigate whether changes in protein constitution occurring during the accelerated aging of detached leaves were similar to those shown previously to occur during the development of attached leaves (Klerk and Van Loon, 1989). Using two-dimensional-polyacrylamide gel electrophoresis (2D-PAGE), the protein patterns of detached oat leaves senescing in either darkness or light were analyzed and compared to those of leaves in which senescence was delayed by application of cytokinin

or enhanced through the action of ABA.

Material and methods

Plant material

Oats (*Avena sativa* L. cv. Victory) was grown in a greenhouse as described previously (Van Loon *et al.*, 1987). Eight days after sowing the 5 cm distal portions of the primary leaves were sampled and incubated vertically in plastic holders with their bases in sterile water or regulator solution (Van Loon *et al.*, 1987). The cytokinin benzyladenine (BA) and abscisic acid (ABA) were used at concentrations of 10 µM and 0.1 mM, respectively. Before extraction the basal 0.5 cm of the segments was cut off and discarded. For comparison, 4.5 cm distal segments of the first leaf attached to 16-days-old plants, which had lost 50% of their maximum protein content, were also harvested.

Extraction of soluble proteins.

Two segments were extracted with 1.5 ml extraction buffer (0.1 M Tris-HCl, 10 mM EDTA, 10 mM 2-mercaptoethanol, 0.1 M NaCl and 0.1% ascorbic acid, pH 7.7) by grinding in a mortar with a pestle with some sea sand and 5% (w/w) polyvinylpyrrolidone at 2 °C. The homogenate was centrifuged and the protein in the resulting supernatant extracted with phenol, precipitated with acetone, centrifuged and dried as described previously (Klerk *et al.*, 1989a).

Gel electrophoretic analysis.

2D-PAGE was performed as described in detail previously (Klerk and Van Loon, 1989a). In all cases, 20 µg of protein was applied to each gel. In the first dimension, isoelectric focusing extended from pH 5 to 8 and a 12.5 % polyacrylamide SDS slab gel was used in the second dimension. Gels were stained with silver as described by Van Telgen and Van Loon (1984), The protein patterns were compared qualitatively using the computer program

GELSCAN (Klerk and Jespers, 1989).

Determination of protein and chlorophyll content

The content of soluble protein was determined according to Bradford (1976) using BSA in the same buffer as a standard. Chlorophyll content was determined as described by Bruinsma (1963).

Results

Rate of senescence under different conditions

In accordance with previous observations (Van Loon *et al.*, 1987), detached leaf segments lost chlorophyll and protein more slowly in the light than in darkness. Under both conditions BA reduced and ABA increased the rate of senescence. The differences between the various conditions are reflected by the incubation times required for 50% loss of protein and of chlorophyll (Table 1). The former times were used as a starting-point to

Table I. Incubation times (days) on different solutions, required for a 50% loss of chlorophyll and protein from leaf segments of 8 days old plants (n=10).

Incubation solution	Light		Darkness	
	Chlorophyll	Protein	Chlorophyll	Protein
water	7	8	5	6
10 µM BA	11	>14	7	10
0.1 mM ABA	4	4	3	4

illustrate the associated effects on the protein patterns. Series of gels were made to follow the course of the alterations in protein patterns during incubation, but only those changes that were consistent and representative for the various conditions are reported.

Changes in protein patterns during incubation in light and darkness

Fig. 1A shows the protein pattern of 8-days-old leaves (t=0). At this stage about 300 spots were clearly discernible. During the natural senescence of attached leaves about 50% of the protein had been lost 16 days after sowing. At this stage about 100 proteins, classified as disappearing spots (Klerk and Van Loon, 1989a) had been lost (Fig. 1B). Accelerated ageing of detached leaves in the light led to an essentially similar pattern, in which minor protein spots rapidly disappeared (Fig. 1C). Furthermore, in both attached and detached leaves spots marked D1, D2 and D3 were readily lost and a pair of spots marked L increased in intensity. Rubisco was still prominent at the stage where 50% of the protein had been lost, and high-molecular-weight proteins synthesized during incipient senescence, marked H (Klerk *et al.*, 1989b) started to disappear an indication that senescence was well advanced (Klerk and Van Loon, 1989b). Whereas senescence-associated proteins, marked N, were present in small amounts in the attached leaves, they had accumulated to readily detectable levels in the detached leaves.

In detached leaves senescing in darkness, the pattern appeared more complex because Rubisco was degraded to a larger extent (Fig. 1D). Proteins D1, D2 and D3 were relatively increased compared to t=0, and so were most of the high-molecular-weight proteins. In addition substantial amounts of the senescence-associated proteins were present. These observations indicate that senescence of detached leaves in darkness differs from that in light in that some proteins appear to be specifically retained.

Changes in protein patterns during incubation on benzyladenine and abscisic acid

During incubation on cytokinin in the light, little protein was lost over a period of two weeks (Table 1). However, even after 7 days some prominent changes were evident in that the D1 proteins had been almost disappeared, the D2 and D3 proteins were reduced and the pair of L proteins had become clearly apparent (Fig. 2A). Thus, these changes seem to be related to the induced ageing rather than to senescence proper. In darkness, particularly the D2 and D3 proteins increased in intensity and no L proteins were apparent. Also, the high-molecular-weight and senescence-associated proteins

were preferentially retained (Fig. 2B), similar to the situation upon incubation in darkness on the water (Fig. 1D). Although BA delayed net protein degradation, the course of senescence was not altered.

Incubation on ABA accelerated senescence but the changes in protein patterns in light and darkness were essentially similar to those on water (Fig. 2C, D). Thus, the regulators did not have significant effects on the alterations in the protein patterns during senescence, whereas specific changes occurred during incubation in light and darkness.

Discussion

As compared to the natural senescence of attached leaves, the accelerated ageing of detached leaves is characterized not only by an enhanced breakdown, but also by a small number of distinct changes in the pattern of soluble proteins. This is particularly evident when comparisons are made at the stage were 50% of the protein has been lost. Whereas the senescence of detached leaves in the light was hardly accelerated as compared to that of attached leaves, and the alterations were comparable, in darkness chlorophyll and protein were more readily lost and specific proteins increased. These comprised both the high-molecular-weight and the senescence-associated proteins, found previously to increase during leaf development (Klerk *et al.*, 1989b), as well as spots disappearing more rapidly during natural senescence (Klerk and Van Loon, 1989a). By following the changes in translatable mRNA's at very early stages of senescence of both attached and excised oat leaves, Malik (1987) found very extensive changes during the first 24 h in darkness. However, most of the initial changes were reversed

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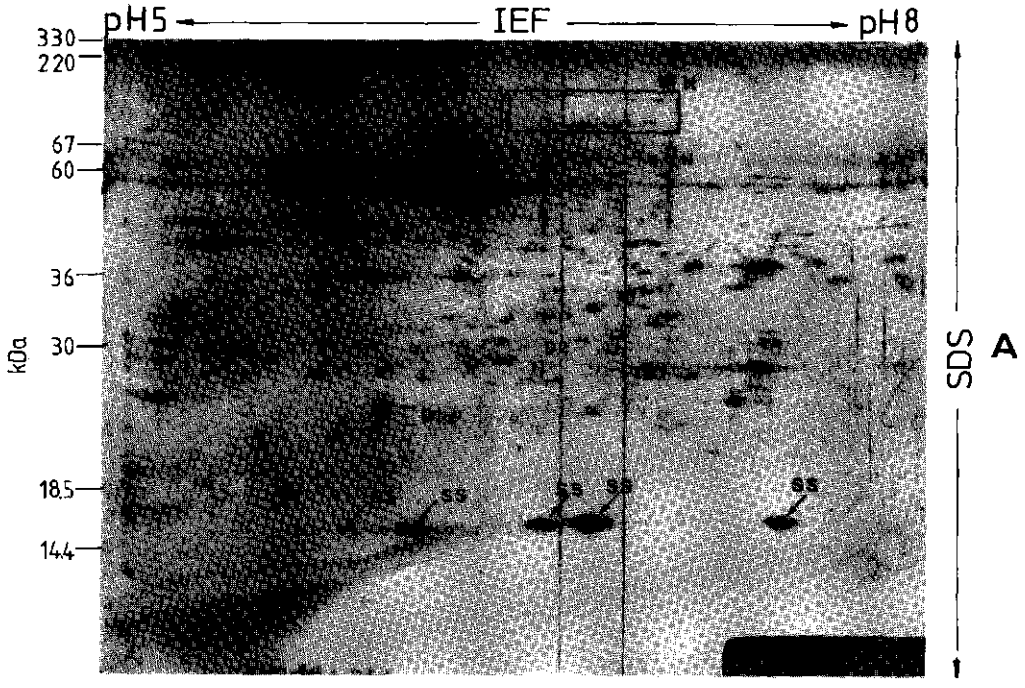
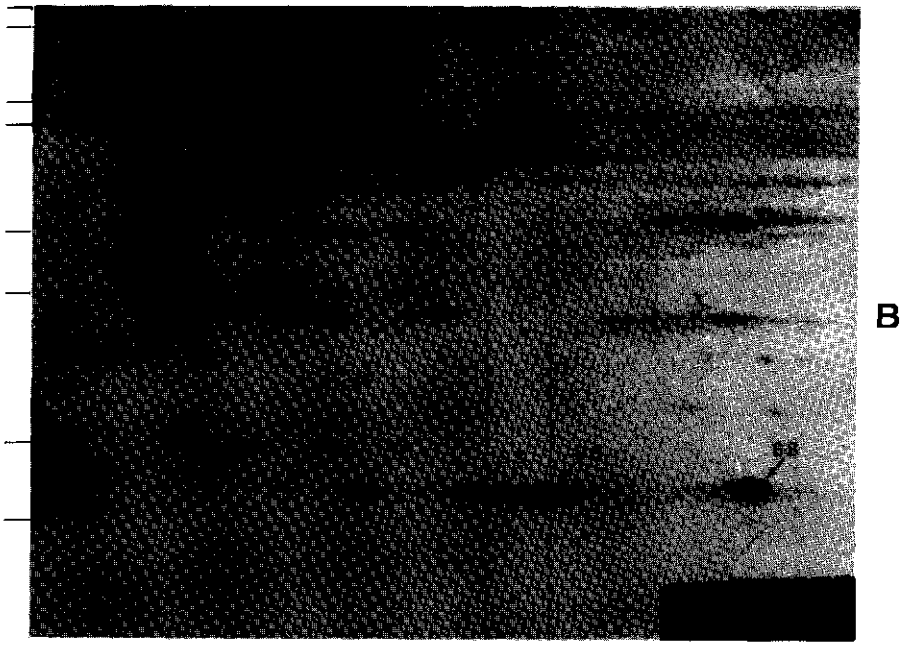
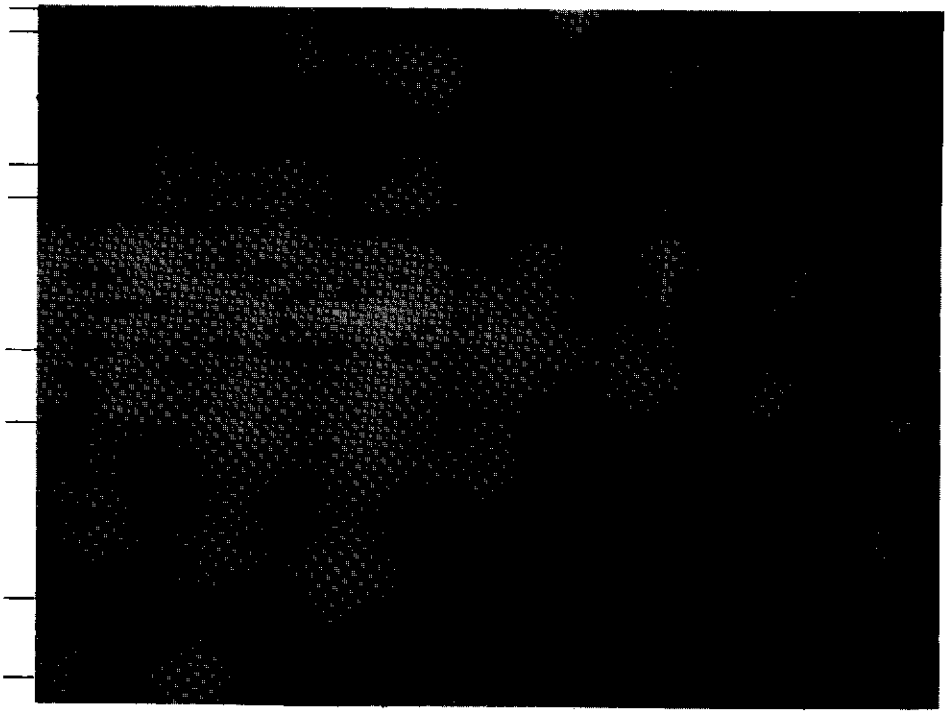
Figure 1 2D-PAGE patterns of soluble proteins from the 4.5 cm distal part of primary oat leaves (A,B) from intact oat plants (A) 8 days or (B) 16 days after sowing and (C,D) detached at day 8 and (C) incubated for 8 days in the light or (D) 6 days in darkness. These times correspond to the stage were 50% of the protein had been lost (Table 1). Samples of 20 µg protein were applied on each gel. The following molecular weight markers were used: thyroglobulin (330 kDa), ferritin (half unit) (220 kDa), BSA (67 kDa), catalase (60 kDa), lactate dehydrogenase (36 kDa), carbonic anhydrase (30 kDa), ferritin (18.5 kDa), α-lactalbumin (14.4 kDa). Gels were stained with silver. H=High-molecular-weight proteins; C=Chloroplast-associated proteins; D1-3= proteins more prominent during incubation in darkness; L= proteins prominent during incubation in light; LS=Large subunit of Rubisco; SS=Small subunit of Rubisco; N=senescence-associated polypeptides.

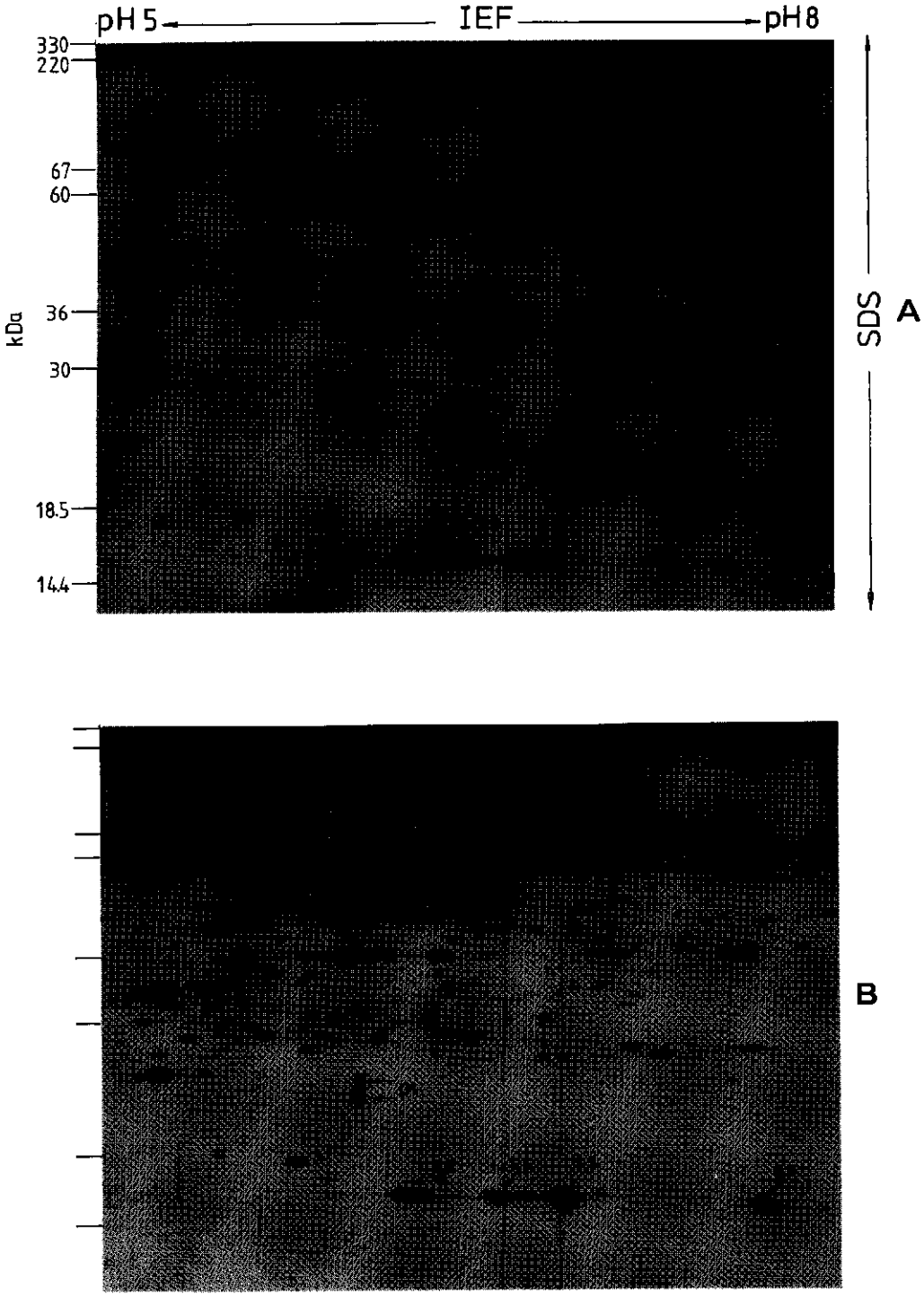

pH5
IEF
pH8
330
220
67
60
36
30
18.5
14.4
kDa
SDS
A


B

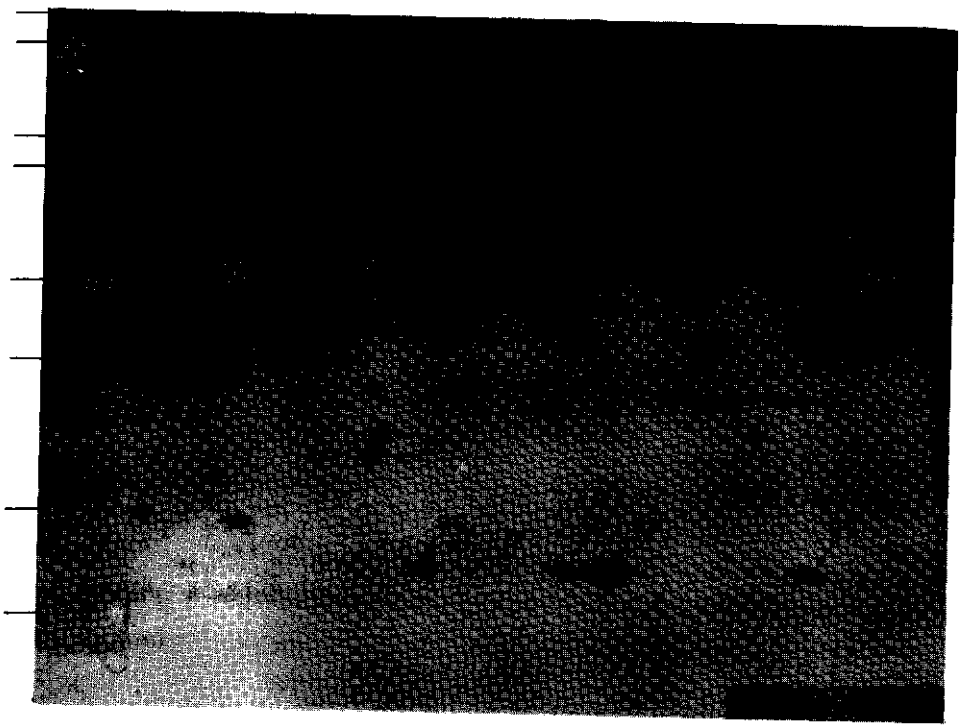


C

D


pH 5
IEF
pH 8
330
220
67
60
36
30
18.5
14.4
kDa
SDS
A
B

C



D

upon return of the leaves to light, indicating that these mRNA's are light-regulated and not necessarily associated with senescence. Moreover, the excised leaf segments were floated horizontally on water, a condition known to give rise to a stress response (Van Loon *et al.*, 1987). Two mRNA's specifically produced during ageing in both attached and detached leaves do not seem to correspond to any of the proteins found to increase in our study. Whereas three mRNA's appeared unique to ageing under light (Malik, 1987), we found two proteins, marked L, that seem to be specific to this condition. The proteins described by Malik (1987) were not found upon *in vivo* labelling of intact plants (Klerk *et al.*, 1989b). These results indicate that the proteins synthesized during *in vitro* translation are not representative of the situation *in vivo*.

Even in light, where it strongly inhibited loss of chlorophyll and protein, BA did not prevent alterations in protein patterns that seem to be related to ageing rather than to senescence. In contrast, in darkness, senescence-related increases in the H and N proteins were substantially more pronounced. In previous studies on protein synthesis (Klerk *et al.*, 1989b) and degradation (Klerk and Van Loon, 1989b) in attached leaves, particularly synthesis of the N proteins was associated with senescence. However, whereas these proteins appeared to turn over rather quickly in attached leaves, in detached leaves they seem to accumulate to readily detectable levels, particularly in darkness, where senescence proceeds most rapidly. Neither kinetin nor ABA led to the occurrence of additional alterations, their effects merely being a retardation and an acceleration, respectively, of the course of senescence on water. Contrary to what was observed recently by Zhi-Yi *et al.* (1988), in darkness ABA did not preserve chlorophyll or protein, but senescence occurred at the same rate as with ABA in the light. The discrepancy may again be ascribed to the use of floating leaf segments in the study of Zhi-Yi *et al.*, (1988).

Whereas the L and some of the D proteins appear to be characteristic of light and darkness, respectively, and do not seem to be related to senescence, the consecutive increase and decrease of high-molecular-weight proteins and the occurrence of the senescence-associated N proteins are typical of senescence progress. Similar changes occurred in attached leaves

Figure 2 2D-PAGE patterns of soluble proteins from the 4.5 cm distal part of leaves, detached on day 8 and incubated (a) for 7 days on BA in the light, (b) for 8 days on BA in darkness, (c) for 4 days on ABA in light and (d) for 3 days on ABA in darkness. Markings as in Fig. 1.

during senescence (Klerk *et al.*, 1989b; Klerk and Van Loon, 1989b). Although the N proteins are synthesized at high levels in senescing leaves (Klerk *et al.*, 1989b), they do not accumulate to any significant level in attached leaves (Klerk and Van Loon, 1989a), apparently as a result of rapid turnover (Klerk and Van Loon, 1989b). Their higher levels in detached leaves in darkness as compared to attached leaves support the hypothesis (Klerk and Van Loon, 1989b) that these proteins may be involved in the senescence process.

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Literature cited

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254.

Bruinsma J 1963 The quantitative analysis of chlorophylls a and b in plant extracts. Photochem Photobiol 2;241-249

Gepstein S, KV Thimann 1981 The role of ethylene in the senescence of oat leaves. Plant Physiol 68: 349-354

Giridhar G, KV Thimann 1985 Interaction between senescence and wounding in oat leaves. Plant Physiol 78: 29-33

Klerk H, A Jespers 1989 GELSCAN, a Personal Computer-program to compare protein patterns on two-dimensional polyacrylamide gels. Chapter 7.

Klerk H, S Tophof and LC Van Loon 1989a Metabolism of proteins in developing leaves of oats (*Avena Sativa* L.). I *In vivo* labelling of the proteins in the first leaf of intact plants. Chapter 3.

Klerk H, S Tophof and LC Van Loon 1989b Metabolism of proteins in developing leaves of oats (*Avena Sativa* L.). II Protein synthesis in the first leaf of intact plants. Chapter 4.

Klerk H, LC Van Loon 1989a Two-dimensional gel electrophoretic changes in soluble proteins during development of primary oat (*Avena sativa* L.) leaves. Chapter 2.

Malik NSA 1987 Senescence in oat leaves: Changes in translatable mRNA's. Physiol Plant 70: 438-446.

Martin C, KV Thimann 1972 The role of protein synthesis in the senescence of leaves. I. The formation of protease. Plant Physiol 49: 64-71

Miller BL, RC Huffaker 1985 Differential induction of endoproteases during senescence of attached and detached oat leaves. Plant Physiol 78; 442-446

Nooden LD, AC Leopold 1980 Phytohormones and the regulation of senescence and abscission. *In* Letham DS, PB Goodwin, TJV Higgins, Eds, Phytohormones and related compounds: A comprehensive treatise. Vol 2 Amsterdam Elsevier. pp329-370

Satler SO, KV Thimann 1979 Relation between leaf senescence and stomatal closure: Senescence in light. Proc Natl Acad Sci USA 76: 2295-2298

Shibaoka H, KV Thimann 1970 Antagonisms between kinetin and amino acids. Experiments on the mode of action of cytokinins. Plant Physiol 46: 212-220

Thimann KV 1980 The senescence of leaves. *In* Thimann KV, Ed, Senescence in plants. CRC Press, Boca Raton. pp 85–115.

Thimann KV, RR Tetley, T Van Thahn 1974 The metabolism of oat leaves during senescence. II Senescence in light. Plant Physiol 59: 448–454

Thomas H, JH Stoddart 1980 Leaf senescence. Ann Rev Plant Physiol 31: 83–111

Thomson JE, RL Legge, RF Barber 1987 The role of free radicals in senescence and wounding. New Phytol 105: 317–344

Udvardy J, GL Farkas, E Marre, E Forti 1967 The effects of sucrose and light on the level of soluble and particle-bound ribonuclease activities in excised *Avena* leaves. Physiol Plant 20: 781–788

Van Loon LC, HCPM Van der Valk, AJ Haverkort, GL Lokhorst 1987 Changes in protease activity in leaves during natural development and accelerated ageing upon detachment. J Plant Physiol 127: 339–353

Van Telgen HJ, LC Van Loon (1984) Two-dimensional gel electrophoretic analysis of chromatin-associated proteins from leaves of different species and varieties of Nicotiana. J Plant Physiol 118: 285–296

Varga A, J Bruinsma 1973 Effects of different cytokinins on senescence of detached leaves. Planta 111:91–93

Woolhouse HW, GI Jenkins 1983 Physiological responses, metabolic changes and regulation during leaf senescence. *In* Dale JE PY Milthorp, Eds, The growth and function of leaves. Cambridge Univ Press. pp 451–487.

Zhi-Yi T, B Veierskov, J Park, KV Thimann 1988 Multiple actions of abscisic acid in senescence of oat leaves. J Plant Growth Regul 7: 213–226

Chapter 7

GELSCAN, a Personal Computer-program to compare protein patterns on two-dimensional polyacrylamide gels

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Summary

Comparing and analyzing a series of two-dimensional gels by hand is troublesome and rather subjective. So far a number of systems for automatic analysis has been developed on mainly mainframe computers, using complex algorithms. This paper presents an inexpensive system, based on a simple Pascal program, to compare individual spots on two-dimensional gels using an IBM- or compatible Personal Computer. The accuracy of the method is demonstrated by comparing two patterns of the same extract from different runs.

Introduction

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) (O'Farrell, 1975) is a powerful technique to analyze complex protein mixtures. By taking advantage of differences in isoelectric point and/or molecular weight of the proteins, these mixtures may be separated in up to 3000 protein spots on a single gel. However, this up to 60-fold increase in resolution over one-dimensional gels also entails problems of pattern analysis. If series of gels have to be compared in order to study, for instance, developmental changes or environmental effects, spots on each gel have to be matched. Furthermore, the great range of abundances and synthetic rates of proteins in cells often dictates that gels containing different amounts of protein be analyzed, or each gel be subjected to a range of autoradiographic exposures. Thus, even with only two patterns per sam-

ple, serial analyses may require 20 or more gels or films, containing on the order of 500 spots or more, to be matched. Only then it is possible to follow the fate of each protein spot under the various conditions studied.

So far a number of systems for automatic analysis have been described (e.g.: Lester *et al.*, 1980; Vo *et al.*, 1981; Lemkin and Lipkin, 1981,1983; Lemkin *et al.*, 1982; Janson *et al.*, 1983; Olson and Miller, 1988). These systems consist of three parts: gel-scanning equipment, a data-storing device, and a program for data comparison. The scanner can be a video camera, a laser scanner, or another optical reader. The storage part can be a hard disk, a magnetic tape drive, etc. For comparing the many data, a mainframe- or minicomputer with a large memory is required. For sufficient resolution and sensitivity, pixels should be as small as possible, preferably not more than 0.1 x 0.1 mm^2. If a gel of 10x20 cm^2 is to be analyzed, one already needs 2.10^6 bits of memory to store this one pattern. Furthermore, when every pixel is attributed a gray value between 0 and 15 as a measure of spot intensity (requiring 4 bits of computer memory), the total amount of memory necessary to store one pattern is about 1 MByte. Under such conditions, comparison of gels on a PC with only 640 kB is impossible.

However, individual spots can be analyzed and matched by comparing the relative positions on two gels. To this end, we have developed an easy and inexpensive method to analyze 2D-gels on an IBM-PC or IBM-compatible computer. This method does not require storage of all the data from a gel but only of the positions of the spots. The system requires a graphic tablet and one IBM-compatible computer with 640 kB of memory and a minimum of one disk drive to store the data on a floppy disk. Using these data as a reference, data from any other gel can be easily compared.

Materials and Methods

Protein extracts and separation

Phenol-soluble leaf proteins from 16-days-old oat plants (*Avena sativa* L. cv. Victory) were extracted and separated by 2D-PAGE on 12.5% polyacrylamide slab gels (120x140x1.5 mm^3) as described elsewhere (Klerk and Van Loon 1989). Reproducible patterns with good resolution and containing in the order of 300 spots, were obtained by applying about 20 µg of protein. Gels

were stained with silver according to Van Telgen and Van Loon (1984).

System configuration

The system was composed of one IBM-compatible computer, one disk drive, one hardcard and 640kB of RAM, and a graphic tablet with a mouse with a red, blue, white and yellow button for data import.

Recording of protein patterns

Stained gels were photographed for permanent records, but analyses were done on 1.4 times enlarged transparent photocopies made directly from the gel using a reproduction camera. All spots were attributed a number and the positions of their center were marked on the photocopy (cf. Fig 2.) Some conspicuous spots present in all the gels to be compared, were selected and matched visually, using the transparencies described above. These matched spots are called marker spots, and are attributed the same identification number in all gels. The program uses these marker spots to correct for size differences or distortions in the patterns. To let the program work efficiently these marker spots must be well distributed over the gel. Whereas the matched marker spots must have the same number in all gels, the further spots to be matched can be attributed arbitrary numbers.

The photocopies were taped on a graphic tablet and the positions of the spots were imported by sequentially pointing to the marked center of each spot with the mouse and pushing the blue or the white button, respectively, to import a matched (marker) or unmatched spot. The session was terminated by pushing the white button. Following the numbered sequence, the coordinates of each spot and its identifying number, as well as a flag to indicate if it is matched or unmatched, were stored in the computer memory. After each session the data were stored on floppy disk for further use.

Matching of protein patterns

With the GELSCAN identifying utility it is possible to compare spots in two gels and match them if their positions are within set limits. One gel

has to be defined as the reference gel (A) and the other as the object gel (B). Spots on the object gel are compared to the previously identified spots on the reference gel. Differences in pharmalytes and acrylamide stock solutions, as well as variation in electrophoresis conditions can generate small but irregular alterations in the protein patterns. Local distortions make it impossible to map all spots on one gel onto a second one with a single transformation. The algorithm we used to solve this problem is a modification of the one described by Vo *et al* (1981), and will be described in detail in the next paragraph.

For any spot to be matched the procedure is as follows:

Step 1. The program selects an unmatched spot in gel B (object gel), searches for several previously defined nearby marker spots in this gel, and selects out of this set three spots which form the smallest possible triangle around the selected unmatched spot in gel B. These marker spots and their counterparts in gel A (reference gel) are then used to perform a transformation for mapping the selected spot on gel B onto gel A.

A spot is considered matched if the transformed center of mass of the spot in gel B and its counterpart in gel A are within a given distance defined by the operator. When more than one spot is found within the critical area, these are all listed.

Step 2. These newly matched spots can now be used reiteratively as further markers to match additional spots. In the default situation the program matches the spots within three reiterative passes. First the program repeats step 2 until it fails to find matchable spots within the critical distance, e.g., 1 mm. After that it repeats the procedure at a different critical distance, e.g., 4 mm. In this last step it uses as markers only those spots that were each previously matched to a single, unambiguously defined spot in the reference gel. All other spots are resubjected to matching until no further matches are found. Whereas the previous pass used only uniquely matched spots as markers, the third pass also works out the best match of spots that could not be unequivocally matched in previous passes.

The accuracy of the final result depends on how numerous and how well distributed the original marker spots are, and as to how far the protein patterns to be matched are distorted.

The algorithm

The first step is to calculate the position of the selected spot to be matched relative to the nearest surrounding marker spots, using the equation:

$$OQ = p(OA) + q(OB) \qquad (1),$$

where Q is the position of the spot to be matched and O, A and B are the positions of marker spots, with OQ, OA and OB the vectors from O to A, B and Q, respectively (Fig. 1, Object gel), and (p,q) are the coordinates of Q with reference to OA and OB as a coordinate system.

The second step is to map an image of Q (designated Q') onto the reference gel. If we assume that the relative position of a particular spot to the same three marker spots in the reference gel is the same as in the object gel, (p,q) will also describe the coordinates of Q' in the reference gel. Designating the three marker spots on the reference gel o, a and b, corresponding to O, A and B in the object gel, respectively (Fig. 1, Reference gel), one then obtains the equation:

$$oQ' = p(oa) + q(ob) \qquad (2).$$

In the third step the program matches Q to the spot nearest to its image Q' within the predefined distances.

The spot editor

The operator can use the spot editor to edit his data. In some cases, the computer lists more than one match for a single spot on the object gel. In other cases, more than one spot in the object gel is matched to the same spot on the reference gel. In the first situation the user must make a choice as to the best match, usually the spot at the smallest distance in the reference gel. In the latter situation the operator has to decide which spot has been correctly identified, and change the name(s) of the other spot(s). After editing, these data can be used to define a new reference gel.

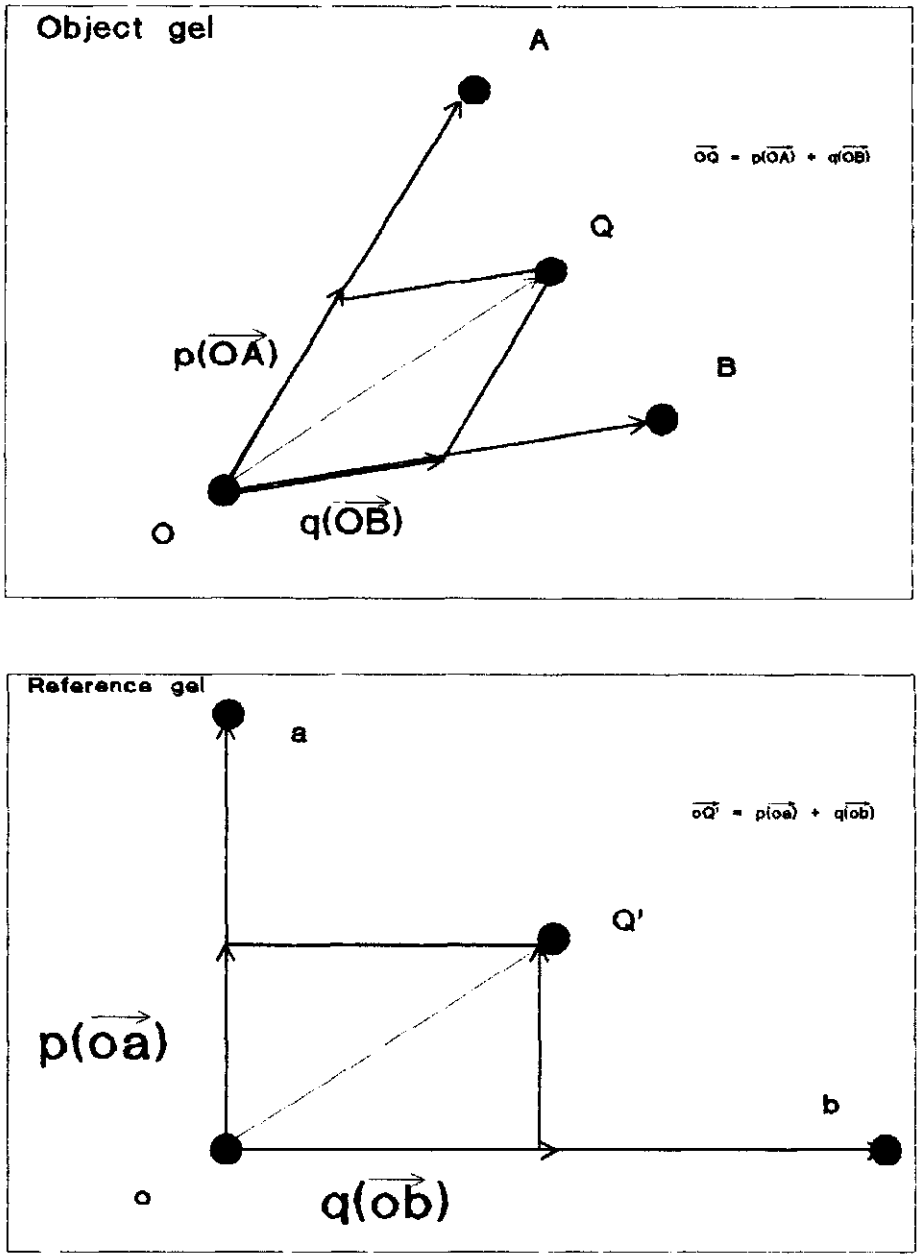


Figure 1. Graphic representation of the equations 1 and 2 described in the text, depicting the coordinate system to determine the position of a particular spot Q in relation to the three marker spots O, A and B.

Table 1. Main Gelscan commands. Spot numbers are numbers given to spots on an object gel by the user; spot names are numbers given by the program after identification with spots on a reference gel. The commands listed here are the most important ones. Commands can be followed by several subcommands (cf. E).

B(Batch) - Make a batch of gels to be specified.
C(Compare)- Compare two specified and identified gels and list differences.
D(Default)- Change default values: Output device (printer or drive), critical distances etc.
E(Editor) - Edit a specified gel.
- Subcommands:
A - Add spot to reference gel.
D - Select first name out of list of alternatives.
d - Select first name and move cursor to next spot.
E - Error mode: cursor jumps automatically to spots with more than one name or with a name already used.
F - Find spot by name.
f - Find spot by number.
L - Measure distance between two specified spots.
M - Select first name and set marker.
m - Set or delete marker.
N - Edit names: del = delete.
enter = select one name.
ins = erase name list and give new name.
P - Print gel data (names bold).
Output to selected device (See Default).
R - Reset markers to original positions.
S - Sort spots by name or number.
W - Create worksheet file. Can be used to plot the positions of the spots.
del - Delete spot.
F(Filter) - Compare object and reference gel and create new gel files, one with the matched spots and one with the non-matched spots.
H(Help) - Gives a list of the commands of the current (sub)menu.
I(Identify)-Identify spots on object gel. After this command the spots are matched with spots on a specified reference gel and the program has given the matched spots one or more names.
L(List) - Gives list of data in memory.
N(Name) - Change name of gel data.
P(Print) - Print or stop current print job.
R(Read) - Read data from disk.
RTN - Read file.
del - Delete file.
esc - Exit to main menu.
N - Change file name.
S(Save) - Save gel data.
Q(Quit) - Quit program.
U(Use) - The user can enter a new set of gel data by bitpad and specify it as reference or object gel.
Y(Erase) - Erase specified gel from memory.

The editor has several functions. Spots can be renamed; spots can be transformed into marker spots; it is possible to make a hard copy of the data, or transfer them into Lotus 1,2,3 worksheet files and plot the positions of the individual spots in a graph. Table 1 lists the various editor commands that are used.

Other functions

Apart from the editor, GELSCAN has several other functions which enable the operator to present data in either tabular or graphic form. Table 1 also lists a summary of those commands.

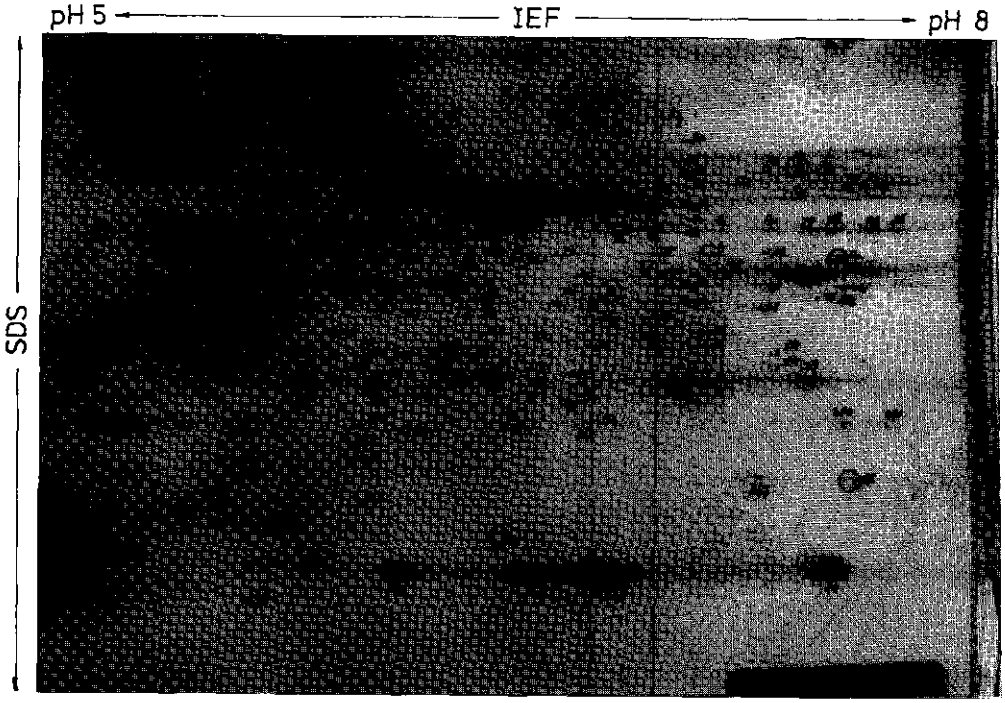
Results

To test the program, two gels of the same protein extract (Fig. 2), but from different runs, were compared. All spots visually identified on both gels were marked and numbered by hand. After introducing 41 marker spots

Table 2. Classification of the 276 spots analyzed from Fig. 2. In the first pass spots were matched within a critical distance of 1mm; in the second pass, the critical distance was set at 4mm A spot was correctly matched when the first choice of GELSCAN was the right spot. Alternatives are spots which were listed in addition to a correct or incorrect match. The incorrect matches and the non-matched spots are specified further in Table 3.

Total number	Number of correct matches		Number of incorrect matches		Not matched
	Without alternative	With alternative	Without alternative	With alternative	
276	249	18	3	4	2
100%	90%	7%	1%	1%	1%

Figure 2. 2D-PAGE patterns from runs of the same preparation of soluble proteins from primary leaves of 16-days-old oats, run at different times. Isoelectric focusing in the first dimension was followed by electrophoresis in a 12.5% polyacrylamide gel containing SDS was used in the second dimension. Fifty µg protein were applied to the gels, and the gels were stained with silver. The spots were numbered such that the same spot (visually identified) has the same number in both gels. The marker spots are encirled. (A) Reference gel; spots marked by an arrow were incorrectly matched or not matched. (B) Object gel; spots marked by an arrow are not matched or incorrectly matched without alternative.



REFERENCE

OBJECT

Table 3. Detailed specification of the incorrectly matched spots from Table 2. The first column specifies the spots that were not correctly matched by the program. These spots are marked with an arrow in Fig. 2A, B. The second column gives the matches proposed, and the third the list of alternatives specified by the program. The right alternative was chosen after visual inspection and is marked by an asterisk.

Spot in object Gel	Match in reference Gel	List of alternatives
54	55	54*
60	61	-
61	63	-
62	64	63; 62*; 65
63	65	64; 63*
64	66	65; 64*
66	67	-
116	-	-
194	-	-

(encircled in Fig. 2) into the program, the spots were matched by GELSCAN using Fig. 2A as the reference gel and Fig. 2B as the object gel. From the total of 276 spots 97% were correctly, 2% incorrectly and 1% not matched at all (Table 2). 7% (18) of the correct matches were not unambiguously assigned and had to be checked by visual inspection of the gel. Of the 7 incorrect matches, 3 were attributed wrong names; for the remaining 4, alternatives were listed, all of which included the correct name (Table 3). These spots are marked by arrows in Fig. 2A and among the listing of alternatives in Table 3 the correct matches are marked by asterisks. The non-matched spots 116 and 194, and the incorrectly matched spots 60, 61, and 66 are near the edge of the gel or in an area without nearby marker spots (cf. Fig. 2B). These situations can explain the poor matching and illustrate the importance of the choice of marker spots to start with.

Discussion

GELSCAN is a tool to help the user compare 2D-gel patterns in a satisfactory way within a time span of less than 1 h. Conditions favoring good results comprise the following:

- The gels to be compared must be as reproducible as possible. It is advisable to run gels simultaneously and to prepare them from the same

solutions. If this is not possible all conditions should be standardized.

- The marker spots must be well distributed. More distorted regions of a gel must contain more marker spots than less distorted ones. Select preferably small, sharp spots as markers. If it is necessary to use big spots be sure to take the center of the spot.

When these conditions are met, GELSCAN functions as described above. However, no program can substitute for the human eye. If one wishes to compare a series of gels which are very similar or show only differences in a known small region of the gel, it may be faster to compare these spots by visual inspection. GELSCAN has been developed to analyze complex patterns where differences may occur in every region of the gel. Although not all spots may be correctly matched, GELSCAN is a powerful tool to compare two sets of spots in an easy and objective way in a relatively short time and with a minimum of extra costs. Only those spots that are not identified directly and unambiguously require further attention. An advantage of GELSCAN is also that it uses simple algorithms written in Turbo Pascal and is divided in different "include" files so that every scientist with some knowledge of the Pascal language can adapt the program to his needs.

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References

Janson PA, LB Grim, JG Elias, EA Bagley, K Lonberg-Holm 1983 Implementation and application of a method to quantitate 2D gel electrophoresis patterns. Electrophoresis 4: 82-91.

Klerk H, LC Van Loon 1989 Two-dimensional gel electrophoretic analysis of the changes in soluble proteins during development of primary oat (*Avena sativa L.*) leaves (Chapter 2.)

Lemkin PF, LE Lipkin 1981 GELLAB: Multiple 2D Electrophoretic gel analysis. Electrophoresis 2: 401-411

Lemkin PF, LE Lipkin 1983. 2-D Electrophoresis gel data base analysis: Aspects of data structures and search strategies in GELLAB. Electrophoresis 4: 71-81

Lemkin PF, LE Lipkin, EP Lester 1982. Some extensions to the GELLAB Two-dimensional Electrophoretic Gel Analysis System. Clin Chem 28: 840-849

Lester EP, P Lemkin, L Lipkin, HL Cooper 1980 Computer-assisted analysis of two-dimensional electrophoreses of human lymphoid cells Clin Chem 26:1392-1403

O'Farrell PH 1975 High resolution two-dimensional electrophoresis of proteins. J Biol Chem 250: 4007-4021

Olson AD, MJ Miller 1988 Elsie 4: quantitative computer analysis of sets of two-dimensional gel electrophoretograms. Anal Biochem 169:49-70

Van Teigen HJ, LC Van Loon 1984 Two-dimensional gel electrophoretic analysis of chromatin-associated proteins from leaves of different species and varieties of Nicotiana. J Plant Physiol 118: 285-296

Vo KP, MJ Miller, EP Geiduschek, C Nielsen, A Olson, NH Xuong 1981. Computer analysis of two-dimensional gels. Anal Biochem 112: 258-271.

Chapter 8

General discussion

Senescence is an integral part of leaf development and is entered after maturity has been reached. Its progress is dependent on proteins synthesis rather than on nucleic acid synthesis (cf Thomas and Stoddart, 1980), suggesting that it may be controlled to a large extent at the posttranscriptional level. Application of phytohormones to leaves on intact plants has little influence on the course of senescence, perhaps because of availability of root-derived cytokinins (Bruinsma, 1980). Whereas upon detachment leaves become subject to accelerated ageing, that can be enhanced and retarded by abscisic acid and benzyladenine, respectively (Chapter 6). The action of these regulators do not involve specific alterations in protein patterns. When the program of senescence is initiated the leaf seems predestined to senesce and eventually die without major changes in morphology or metabolism, unlike the burst of activity occurring during the ripening of e.g. climateric fruits (cf. Woolhouse, 1978). Regulators can only enhance or retard leaf senescence, but the developmental course of senescence is not changed.

In view of the requirement for protein synthesis, it was attempted to relate the various stages in the life of the first leaf of oats to alterations in protein patterns. Martin and Thimann (1972) originally suggested that the net degradation of protein during senescence was controlled by increases in the activities of major proteases. However, Van Loon *et al.* (1987) have shown that synthesis of additional proteases is not required. Thus, the question was again raised which proteins need to be synthesized for senescence to proceed. Since inhibition of protein synthesis on cytoplasmic ribosomes is far more efficient in inhibiting loss of protein and chlorophyll than inhibition of protein synthesis on organellar ribosomes, and cellular compartmentation is maintained into a late stage of senescence (Thomas and Stoddart, 1980), this study has concentrated on soluble proteins.

To avoid the complications of wounding, stress and accelerated ageing inherent in the use of detached leaves, development was followed of attached leaves of intact plants. To be able to study protein metabolism under these conditions, a method was devised which allowed efficient uptake of

labelled precursors and incorporation into protein. Thus, it was possible to study protein synthesis directly rather than having to rely on *in vitro* translation of mRNA. A comparison of the 2D-PAGE patterns of *in vitro* translated proteins from oat leaves, as described by Malik (1987), with our patterns of *in vivo* protein synthesis, shows little similarity, indicating that the former does not reliably reflect the *in vivo* situation. The differences may be due to different extents of translation of mRNA's *in vitro* and *in vivo*, varying mRNA stability, various posttranslational modifications of the primary translation product *in vivo*, and differential protein turnover. Moreover, the conclusion that synthesis of proteins necessary for senescence to proceed, is regulated posttranscriptionally makes one to expect to see differences in *in vivo* synthesized proteins, that are unlikely to be evident upon *in vitro* translation.

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) was used to investigate whether particular proteins are characteristic of certain stages of leaf development, and whether especially specific proteins can be found that are linked to the senescence stage. Both by protein staining and by labelling and fluorography many proteins were identified that were lost once leaves had expanded to their final length, but no proteins were found to be present or synthesized exclusively at the later stage of leaf development. Nevertheless, a few proteins were apparent, whose synthesis is gradually and continuously enhanced with leaf age. Besides changes identified in leaves senescing on the plant, detached leaves showed alterations that reflected their condition of incubation rather than effects associated with senescence. Thus, it was concluded that detached leaves are indeed less suitable for the study of senescence than leaves attached to the plant.

By using 2D-PAGE O'Farrell (1975) detected about 1100 proteins in *Escherichia coli*. Since then up to 3000 different proteins have been identified in mammalian cells (Celis and Bravo, 1983). Calculations by Kamalay and Goldberg (1980) have indicated that in tobacco leaves about 27,000 are expressed, indicating that more proteins must be present than can be resolved by the present technique. The maximum number of proteins detectable in the first leaf of oats was only 500. This number is fairly representative of 2D-PAGE patterns of plant proteins in general (e.g. Perras and Sarhan, 1989). One reason could be that a large number of proteins has a pI outside the interval selected. This explanation does not seem valid, because in chapter 1 I demonstrated that the majority of the proteins has a

pI between pH 5 and 8. Another, more plausible explanation is that in plants the proteins differ in amount far more than in, for instance, bacteria. In *E. coli* most protein spots show about the same size and intensity, whereas in oat leaves some proteins are present in large amounts (e.g., Rubisco), while others are barely detectable. When an optimal amount of protein is applied to the gel it is separated equally over a large number of spots in *E. coli* whereas in oat leaves the largest amount of protein is represented by a small number of major spots. Thus it is quite possible that minor proteins, characteristic of the various stages of leaf development, have escaped detection.

The possibility that leaf senescence is initiated as a result of a gradual decline of protein synthesis with degradation taking over, has also to be considered. Rubisco and other chloroplast proteins are synthesized at an early stage during leaf development (Chapter 4). Particularly Rubisco synthesis stopped during the early stage of senescence (Chapter 4), but the protein could still be detected in very old plants (Chapter 2 and 5). In maize leaves its half-life has been estimated 6-7 days (Simpson *et al.*, 1981). In our study, the protein disappeared only gradually from the obtained patterns and no accelerated degradation in the later stage of senescence was apparent (Chapter 2 and 5). Even though the incorporation of ^{35}-methionine was reduced at the later stages of senescence, a great many proteins, among which also chloroplast proteins, were still being synthesized (Chapter 4). Thus, although the rate of protein synthesis declines with leaf age and the total amount of protein is reduced, the leaf seems to retain the capacity for functioning well into an advanced stage of senescence.

No specific properties of the proteins, as identified by 2D-PAGE, acted as determinants of their rate of degradation. As shown in Chapter 5, the pattern of protein degradation was quite similar early and later in leaf development. Thus, the mechanisms governing protein turnover do not seem to change at the onset of senescence. This conclusion is in full accord with previous observations by Van der Valk and Van Loon (1988), that the major acidic protease remains compartmentalized in the vacuole until at least an advanced stage of senescence. Because of the different patterns of degradation of individual proteins in cells and in cell-free extracts, senescence can not be caused by a gradual leakage of the acid protease out of the vacuole or result from the activity of a simple neutral protease in the cytosol. Probably both proteases are confined to lysogenic compartments as

defined by Matile (1975). This might be the vacuoom, a space allegedly connecting extracellular space, vacuolar space and intermembrane space surrounding mitochondria and chloroplasts . Under such conditions the vacuolar protease could easily be transported to any of these subspaces. Chloroplast proteins could then be degraded by the acidic protease in the intermembrane space. The neutral protease could function in different compartments with a neutral pH.

Recent studies indicated the occurrence of several minor proteases in chloroplasts (Waters *et al.*, 1982; Martinoia *et al.*, 1983; Thayer *et al.*, 1988) and mitochondia (Chua and Schmidt, 1979). It is not clear whether these proteases function in organellar protein breakdown during senescence or in normal turnover. These functions are not mutually exclusive, however, continuous turnover of e.g. chloroplast proteins in the absence of compensating synthesis would gradually deplete the organelle of proteins. Essentially, this is what is observed when one takes into account the much slower loss of proteins *in vivo* as compared to *in vitro*. Thus, final loss of function seems to be the inevitable outcome of a gradual loss of synthetic capacity, coupled to a sustained or more slowly declining level of protein breakdown.

The regulation of protein metabolism in developing leaves must be more complicated however, because protein synthesis is also required for progression of the leaf through the phase of senescence. As shown in chapter 4, specific high-molecular-weight proteins (H) show an enhanced synthesis during the onset and progression of senescence. Particularly the three N proteins were synthesized in progressively larger amounts at later stages of senescence. It is not inconceivable that at the start of senescence, senescence-associated mRNA's (cf. Malik, 1987) are synthesized and that senescence is regulated by a consecutive enhancement of translation of subsets of the relatively stable mRNA's with leaf age. This could explain that inhibitors of translation, not of transcription, affect senescence in most cases (Thomas, 1975; Von Abrams, 1974; Makovetzki and Goldsmidth, 1976; Martin and Thimann 1972). It was shown in Chapters 4 and 5 that particularly the N proteins are turning over rapidly, but still accumulate to readily detectable levels in detached leaves that senesce more rapidly than attached leaves. Thus, the progression of senescence may be associated with continuous synthesis of a set of rapidly turning-over high-molecular-weight proteins. Apart from their molecular weights (67 kDa) and pI values (6.5-6.8), no properties of these proteins are known and their function remains

to be established.

References

Bruinsma 1980 Hormonal regulation of senescence, ageing, fading, and ripening. *In* Lieberman H, Ed, post-harvest physiology and crop preservation. Plenum press, New York, pp 141-163

Celis J, R Bravo (eds) 1983 Two-dimensional gel electrophoresis of protein. Academic Press, San Diego, USA.

Chua NH, GJ Schmidt 1979 Transport of proteins into mitochondria and chloroplasts. J Cell Biol 81: 461-483

Kamalay JC RB Goldberg 1980 Regulation of structural gene expression in tobacco. Cell 19:935-946

Makovetzki S, EE Goldsmidth 1976 A requirement for cytoplasmic protein synthesis during chloroplast senescence in aquatic plant *Anacharis canadensis*. Plant Cell Physiol 17: 859-862

Malik NSA 1987 Senescence in oat leaves: Changes of translatable mRNA's Physiol Plant 70: 438-446

Martin C, KV Thimann 1972 The role of protein synthesis in the senescence of leaves. I, the formation of proteases. Plant Physiol 49: 64-71

Martinoia E, U Heck, MJ Dalling, P Matile 1083 Changes in chloroplast number and chloroplast constituents in senescing barley leaves. Biochem Physiol Planzen 178:147-155

Matile P 1975 The lytic compartment of plant cells. Cell Biology Monographs, Vol I. Springer Verlag Wien, New York.

O'Farrell PH 1975 High resolution two-dimensional electrophoresis of proteins. J Biol Chem 250:4007-4021

Perras M, Sarhan F 1989 Synthesis of freezing tolerance proteins in leaves, crown and roots during cold acclimation of wheat. Plant Physiol 89:577-585

Simpson E, Cooke RJ, Davies DD 1981 Measurement of protein degradation in leaves of Zea Mays using [^{3}H] acetic anhydride and tritiated water. plant Physiol 67: 1214-1219

Thayer SS, HT Choe, S Rausser, RC Huffaker 1988 Characterization and subcellular localization of amino peptidases in senescing barley leaves. Plant Physiol 87:894-897

Thimann KV 1980 The senescence of leaves. *In* Thimann KV, Ed, senescence in

plants. KV CRC Press, Bocta Raton. pp. 85-115

Thomas H 1975 Regulation of alanine aminotransferase in leaves of *Lolium temulentum* during senescence. Z Plantenphysiol 74: 208-218

Thomas H, Stoddart JL 1980 Leaf senescence. Ann Rev Physiol 31:83-111

Van der Valk HCPM, van Loon LC 1988 Subcellular localization of protease in developing leaves of oats (*Avena sativa L.*). Plant Physiol 87:536-543

Van Loon LC, Van der Valk HCPM, Haverkort AJ, Lokhorst GL 1987 Changes in protease activity in leaves during natural development and accelerated ageing upon detachment. J Plant Physiol 127:339-353

Von Abrams GJ 1974 An effect of ornitine on degradation of chlorophyll and protein in excised leaf tissue. Z Plantenphysiol 72: 410-421

Waters SP, RE Noble, MJ Dalling 1982 Intracellular localization of peptide hydrolases in wheat (*Triticum aestivum* L.) leaves. Plant Physiol 68:575-579

Woolhouse HW 1978 Senescence Processes in the Life Cycle of flowering plants. Bioscience 28:25-31

Summary

The development of the first leaf of oats (*Avena sativa* L.) comprises four phases. About 4 day after sowing the leaf emerges and starts to expand. Expansion is complete and maturity is reached at 7 to 9 days. Thereafter, senescence ensues, as expressed by a gradual loss of chlorophyll over the period from 12 to 37 days. Finally the leaf withers, and dies by about 37 days.

RNA and protein contents start to decrease before the onset of chlorophyll loss, and during the phase of senescence all three parameters decline in a coordinated manner. Since proteins are essential to the functioning of the leaf, their loss is considered to be a determining factor in the rate at which senescence proceeds. However, inhibitors of protein synthesis have been shown to block loss of protein and chlorophyll, indicating that also synthesis of proteins is required for senescence to progress. These proteins are not major proteases, nor are they likely to be membrane-associated, because membrane systems remain intact until a late stage of senescence.

The present study was undertaken to search for proteins that are specific to each of the developmental stages in the life of the leaf, and to determine specific properties of proteins that function particularly during the stage of senescence. Changes in the pattern of soluble proteins during leaf development were followed by using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) (Chapter 2). Phenol-soluble protein samples were separated in up to 500 spots by isoelectric focusing between pH 5 and 8 in the first dimension and 12.5% SDS slab gel electrophoresis in the second dimension. The gels were stained with silver. A number of 122 protein spots were further characterized by their presence in isolated chloroplasts. The large and the small subunits of ribulosebisphosphate carboxylase/oxigenase (Rubisco) were identified by immunoblotting.

Major changes occurred during the period of leaf expansion: 39 mostly low-molecular-weight polypeptides disappeared and 34 mostly high-molecular-weight spots became apparent. Of these, 16 and 13, respectively, were associated with chloroplasts. During the subsequent loss of protein and chlorophyll, indicative of senescence, the number of spots decreased gradually without new polypeptides becoming apparent, 75% of the total number being still detectable in yellow, 37 days-old leaves. About half of the disappearing spots were lost before the leaf had expanded to its final

length. No polypeptides were detected that were present exclusively in the later stages of leaf development: maturity, senescence, and final collapse.

It was considered that such proteins might be present in amounts below the limit of detection by general protein staining, either because they are synthesized in very small amounts, or because they are subject to rapid turnover. To be able to follow the synthesis and degradation of individual proteins, while avoiding effects of leaf detachment or wounding, a method was developed to label the proteins at different stages of development of intact leaves attached to the plants (Chapter 3). The following methods were tested: growth of seedlings on ^{35}S-sulfate-containing Knop medium, labelling with ^{35}S-methionine by vacuum infiltration of the leaf, injection into the leaf base or into the seed near the embryo, or wiping the surface of the leaf with ethanol and subsequent incubation in the labelled solution. The first four methods were unsuitable because of insufficient uptake, preferential transport to other plant parts, too much dilution by endogenous aminoacids or radiation damage. A specific activity of 10^5 dpm / 20 µg of leaf protein, minimally necessary to obtain a distinct fluorogram of the gels, was reached only upon the treatment with ethanol. Under these conditions no more than 170 kBq of ^{35}S-methionine was required in the uptake solution.

When the 4.5 cm distal parts of the first leaf were thus labelled for 12 h at different stages of development, similar amounts of label were retained in the segments, but incorporation into protein decreased from 28% in 7-days- to 3% in 27-days-old plants. Even at a late stage of senescence a great many proteins were still being synthesized. Synthesis of Rubisco and the other chloroplast-associated proteins declined more rapidly than general protein synthesis. Although Rubisco ceased to be synthesized by 13 days, it was still clearly present in 27-days-old leaves indicating that, once synthesized, the protein is very stable. During senescence two sets of relatively high-molecular-weight proteins became more pronounced. In addition, three proteins with a molecular weight around 67 kDa and isoelectric points between 6.5 and 6.8 became the most prominently synthesized proteins in older leaves. Since these senescence-associated proteins were hardly visible on silver-stained patterns, they seem to be subject to rapid turnover. A functioning of these proteins in senescence could explain the requirement of protein synthesis for senescence to proceed (Chapter 4).

By labelling distal leaf parts of 7- and 15-days-old plants for 24 h and following the loss of label from individual protein spots, the rates of

degradation of the synthesized proteins were determined over a 14-day period. Of the about 300 spots that could be distinguished, a large number turned over quickly (disappearing spots), while a smaller number was degraded very slowly (persisting spots). Thus, protein half-lifes varied from a few days to over at least a week. This pattern of *in vivo* breakdown was compared to that *in vitro* in soluble-protein extracts at pH 5.5 and 7.5, the optimal pH values of the acidic and neutral proteases, respectively. Although 75% of the proteins were degraded similarly *in vivo* and *in vitro*, substantial changes in the relative rates of degradation of the other proteins occurred under the different conditions. Notably, Rubisco was degraded very slowly *in vivo*, but rather quickly and in a different way *in vitro* at pH 5.5. As it has been found that there is no relationship between the level of protease activity and the rate of protein degradation *in vivo*, these results support our previous conclusion (Van der Valk and Van Loon 1988) that the proteases and their protein substrates are spatially separated *in vivo*. Whereas rapidly turning-over cytoplasmic proteins might be imported into the vacuole for degradation, long-lived chloroplastic proteins, such as Rubisco, are likely to be degraded by proteases within the organelle.

To investigate in how far the changes observed in attached leaves also occur upon detachment, distal leaf segments were incubated vertically with their bases in water. Senescence of detached leaves in light did not differ significantly from senescence in attached leaves. In darkness, protein was lost at a higher rate, but several proteins showed relative increases. Notably, proteins previously characterized as high-molecular-weight and senescence-associated proteins increased in amount. Additional changes associated with incubation in light or darkness were not related to senescence. The cytokinin benzyladenine delayed and abscisic acid accelerated the changes compared to water. Besides changes previously identified in leaves senescing on the plant, detached leaves showed alterations that reflect their condition of incubation rather than their developmental stage. For this reason, detached leaves are less suitable for the study of senescence than leaves attached to the plant (Chapter 6).

The comparison of many protein patterns was facilitated by the development of a computer program, GELSCAN, for the qualitative matching of any pair of gels. The program is written in PASCAL and makes use of a 640 kB personal computer. This program is a valuable tool for an objective analysis of 2D-PAGE patterns (Chapter 7).

Finally, the regulation of protein synthesis and degradation during development of the first leaf of oats is discussed (Chapter 8). No specific properties of the proteins themselves, as identified by 2D-PAGE, act as determinants of their rate of degradation. The high-molecular-weight proteins, and particularly the senescence-associated proteins, are synthesized in increasing amounts during senescence and may play a role in mechanisms governing the senescence progress.

References

Van der Valk HPCM, LC Van Loon 1988 Subcellular localization of protease in developing leaves of oats (*Avena sativa* L.). Plant Physiol 87:536-543

Samenvatting

De ontwikkeling van het eerste blad van haver (*Avena sativa* L.) is te verdelen in 4 fasen. Vier dagen na het zaaien komt het blad te voorschijn en begint te strekken, totdat het tussen 7 en 9 dagen volgroeid is. Daarna begint het blad te verouderen, hetgeen zich uit in een geleidelijk verlies van chlorofyl over een periode van 12 tot 37 dagen. Tenslotte verwelkt het blad, droogt uit en sterft af rond dag 37.

Voordat chlorofylverlies optreedt beginnen de hoeveelheden RNA en eiwit in het blad al af te nemen. Tijdens de verouderingsfase nemen RNA, eiwit en chlorofyl alle drie evenredig af. Omdat eiwitten essentieel zijn voor het functioneren van het blad, wordt het verlies van eiwit gezien als een bepalende factor voor de snelheid waarmee de veroudering voortschrijdt. Er is echter aangetoond dat remming van de eiwitsynthese gedurende de bladveroudering het verlies van zowel eiwit als chlorofyl remt. Dit wijst er op dat ook eiwitsynthese nodig is voor bladveroudering. Deze nieuw gesynthetiseerde eiwitten kunnen niet de kwalitatief voornaamste proteasen zijn. Ook is het niet waarschijnlijk, dat deze eiwitten membraangebonden zijn omdat de membraansystemen in de bladcellen tot in een laat stadium van de veroudering intact blijven.

In het onderzoek dat in dit proefschrift beschreven wordt, is er gezocht naar eiwitten die specifiek zijn voor elk van de ontwikkelingsstadia tijdens het leven van een blad. Daarnaast is getracht specifieke eigenschappen te definiëren van eiwitten, in het bijzonder voor het functioneren tijdens het verouderingsstadium. Veranderingen in het patroon van de oplosbare eiwitten gedurende de bladontwikkeling werden vervolgd met behulp van tweedimensionale polyacrylamide gel elektroforese (2D-PAGE) (Hoofdstuk 2.). Hierbij worden eiwitten eerst in een pijpgel gescheiden op grond van hun isoelektrisch punt en vervolgens dwars daarop in een slabgel op grond van hun molecuulgewicht. Na extractie uit het bladweefsel werden de eiwitten in het extract overgebracht in een fenol-bevattende fase, neergeslagen met aceton en opgenomen in buffer. De eiwitmonsters werden gescheiden in tot 500 spots, door middel van isoelektrische focussering tussen pH 5 and 8 in de eerste dimensie en elektroforese in een 12.5% SDS slab gel in de tweede dimensie. Daarna werden de gels gekleurd werden met zilver. 122 spots werden verder gekarakteriseerd als eiwitten geassocieerd met chloroplasten. De grote en de kleine ondereenheid van ribulosebisfosfaatcarboxylase/oxigenase werden geïdentificeerd met behulp van immunoblotting.

Grote veranderingen traden op gedurende de laatse fase van de bladgroei: 39 merendeels laagmoleculaire eiwitten verdwenen, terwijl ter zelfder tijd 34 merendeels hoogmoleculaire eiwitten verschenen. Daarvan waren er respectievelijk 16 en 13 geassocieerd met chloroplasten. Gedurende het daaropvolgend verlies van eiwit en chlorofyl tijdens de veroudering verminderde het aantal spots geleidelijk zonder dat nieuwe eiwitten verschenen. Niettemin was 75% van het totale aantal spots nog aantoonbaar in gele 37 dagen oude bladeren. Ongeveer de helft van de verdwijnende spots was al verdwenen voordat het blad zijn uiteindelijke lengte had bereikt. Er konden geen eiwitten worden aangetoond die uitsluitend voorkwamen in één van de latere stadia van de bladontwikkeling: Volgroeid, verouderend en afstervend.

Het werd mogelijk geacht dat dergelijke eiwitten in zo kleine hoeveelheden aanwezig zijn dat zij niet konden worden gedetecteerd met de algemene eiwit-kleuringsmethoden. Dit kan het geval zijn omdat zij in zeer geringe hoeveelheden gesynthetiseerd worden of omdat zij een hoge "turnover" hebben, d.w.z. dat zij snel worden gesynthetiseerd maar ook weer snel worden afgebroken. Om de synthese en afbraak van individuele eiwitten te kunnen vervolgen, maar tegelijk de effecten van afsnijden en/of verwonding te vermijden, werd een methode ontwikkeld om de eiwitten in intacte bladeren aan gehele planten in verschillende ontwikkelingsfasen te merken met een radioactieve precursor (Hoofdstuk 3). De volgende methoden werden getest: groei van zaailingen op ^{35}S-sulfaat bevattend Knop medium, merken met ^{35}S-methionine door middel van vacuuminfiltratie van het blad, injectie in de bladbasis of in het zaad vlakbij het embryo, of door de oppervlakte van het blad te bestrijken met ethanol en vervolgens de behandelde bladgedeelten te incuberen in een radioactieve oplossing. De eerste vier methoden bleken ongeschikt als gevolg van onvoldoende opname van de label, preferentieel transport naar andere delen van de plant, verdunning door endogene aminozuren of door stralingsschade aan het groeiende kiemplantje. Een specifieke activiteit van 10^5 dpm / 20 µg eiwit, minimaal nodig voor het verkrijgen van duidelijke fluorogrammen van de gels, werd alleen bereikt na de behandeling met ethanol. Onder deze condities was 170 kBq ^{35}S-methionine in de opname-oplossing voldoende om dit niveau te bereiken.

Wanneer op deze wijze 4,5 cm van de top van het eerste blad in verschillende ontwikkelingsstadia, zo gedurende 12 h werd gelabeld, werden vergelijkbare hoeveelheden radioactiviteit opgenomen, maar de inbouw in eiwit daalde van 28% in 7 dagen oude bladeren tot 3% in 27 dagen oude bladeren. Zelfs in een laat stadium van de veroudering werd nog een groot aantal

eiwitten gesynthetiseerd. De synthese van Rubisco en andere met de chloroplast geassocieerde eiwitten verminderde sneller dan de totale eiwitsynthese. Hoewel Rubisco al 13 dagen na zaaien niet meer werd gesynthetiseerd, was dit eiwit nog duidelijk aanwezig in 27 dagen oude bladeren. Dit wijst er op dat het eenmaal gesynthetiseerde eiwit uiterst stabiel is. Gedurende de veroudering traden twee groepen van hoog-moleculaire eiwitten meer op de voorgrond. In een later stadium werd een derde groep van drie eiwitten met een molecuulgewicht van 67 kDa en een isoelectrisch punt tussen de 6.5 en 6.8 het meest opvallend onder de gesynthetiseerde eiwitten. Omdat deze met veroudering geassocieerde eiwitten nauwelijks zichtbaar waren op met zilver gekleurde gels, is het waarschijnlijk dat zij een hoge turnover hebben. Een betrokken zijn van deze eiwitten bij de veroudering zou een verklaring kunnen vormen voor de noodzaak van eiwitsynthese voor de voortgang van de veroudering (Hoofdstuk 4).

Door de bladtoppen van 7 en 15 dagen oude planten gedurende 24 uur te labelen en vervolgens het verlies van radioactiviteit uit de afzonderlijke eiwitspots te vervolgen, werden de afbraaksnelheden van de gesynthetiseerde eiwitten gedurende een periode van 14 dagen bepaald. Van de ongeveer 300 spots die konden worden onderscheiden, vertoonde een groot aantal een snelle turnover (verdwijnende spots), terwijl een kleiner aantal zeer langzaam werd afgebroken (blijvende spots). Hieruit volgt dat de halfwaardetijd van deze eiwitten uiteenliep van enkele dagen tot meer dan een week. Dit patroon van afbraak *in vivo* werd vergeleken met dat *in vitro*, in eiwitextracten bij pH 5.5 en 7.5, de optimale pH van respectievelijk de zure en de neutrale proteasen. Hoewel 75% van de eiwitten *in vivo* en *in vitro* gelijkelijk werden afgebroken, waren er onder de verschillende condities aanzienlijke verschillen in de relative afbraaksnelheden van de resterende eiwitten. In het bijzonder Rubisco werd *in vivo* zeer langzaam afgebroken maar tamelijk snel en op een andere wijze *in vitro* by pH 5.5. Aangezien vastgesteld is dat er geen verband bestaat tussen de hoogte van de proteaseactiviteit en de snelheid van eiwitafbraak *in vivo*, ondersteunen vorige conclusies (Van der Valk and Van Loon, 1988) dat de proteasen en hun eiwit substraten ruimetelijk zijn gescheiden. Terwijl cytosolische eiwitten met een hoge turnover wellicht kunnen worden afgebroken als gevolg van transport in de vacuole, is het mogelijk dat langlevende eiwitten in de chloroplast, zoals Rubisco, afgebroken worden door specifieke proteasen die in kleine hoeveelheden in de chloroplast aanwezig zijn.

Om te onderzoeken in hoeverre de veranderingen, die worden waargenomen in

intacte bladeren, zich ook voordoen in afgesneden bladeren, werden bladtoppen afgesneden en verticaal geïncubeerd met hun snijvlak in water. Veroudering van afgesneden bladeren in het licht verschilde weinig van de veroudering van bladeren aan intacte planten. In het donker verloren de bladsegmenten hun eiwit aanzienlijk sneller maar verscheidene eiwitten vertoonden een relatieve toename. In het bijzonder de hierboven beschreven hoog-moleculaire en de met de bladveroudering geassocieerde eiwitten namen in hoeveelheid toe. Verdere veranderingen waren geassocieerd met de incubatie in het licht of donker en hadden geen betrekking op de optredende veroudering. Het cytokinine benzyladenine remde, en abscisinezuur versnelde de veranderingen in het eiwitgehalte vergeleken met water. Naast de eerder beschreven veranderingen die zich voordeden in natuurlijk verouderende bladeren, vertoonden afgesneden bladeren veranderingen die meer het gevolg waren van de incubatie-omstandigheden dan van de optredende veroudering. Om deze reden zijn afgesneden bladeren minder geschikt voor verouderingsonderzoek dan bladeren aan de plant (Hoofdstuk 6).

Vergelijking van de vele 2D-PAGE-patronen werd vergemakkelijkt door de ontwikkeling van het computerprogramma GELSCAN. Dit programma vergelijkt de positie van individuele spots in twee patronen. Het programma is geschreven in Pascal en maakt gebruik van een 640 kB "personal computer". Dit programma is een waardevol hulpmiddel voor een analyse van 2D-PAGE patronen.

In het laatste hoofdstuk (Hoofdstuk 8) wordt de regulatie van de eiwitsynthese en -afbraak gedurende de bladontwikkeling bediscussieerd. Er werden geen specifieke eigenschappen zoals lading en molecuulgrootte gevonden, die een rol zouden kunnen spelen bij de afbraaksnelheid van eiwitten *in vivo*. De hoogmoleculaire eiwitten en in het bijzonder de speciaal de met veroudering geassocieerde eiwitten worden tijdens veroudering in steeds sterkere mate gesynthetiseerd en spelen mogelijk een rol in mechanismen die de voortgang van het verouderingsproces bepalen.

Literatuur

Van der Valk HPCM, LC Van Loon 1988 Subcellular localization of protease in developing leaves of oats (*Avena sativa* L.). Plant Physiol 87:536-543

Curriculum Vitae

Hans Klerk werd geboren op 25 juli 1956 te Koog aan de Zaan. Na het behalen van het Atheneum B diploma, via MAVO en HAVO, ging hij in 1977 Biologie studeren aan de Universiteit van Amsterdam. In 1983 behaalde hij het doctoraal examen met als hoofdvak Plantenfysiologie en als bijvakken Aquatische Oecologie en Biochemie. In 1984 begon hij, na een tijdelijke baan als wetenschappelijk medewerker en een periode van vrijwilligerswerk, beide bij de Vakgroep Plantenfysiologie van de Universiteit van Amsterdam, aan het promotie-onderzoek op de vakgroep Plantenfysiologie van de Landbouwuniversiteit te Wageningen, hetgeen resulteerde in dit proefschrift. Sinds december 1987 werkt hij als plantenfysioloog / biochemicus bij de vakgroep Biochemie van de Landbouwuniversiteit te Wageningen.