CENTRALE LANDBOUWCATALOGUS
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Changes in chromatin-associated proteins of virus-infected tobacco leaves

Proefschrift
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Stellingen

1. Binding van door een virus gecodeerd eiwit aan het DNA van zijn gastheer vormt één van de strategieën die virussen in staat stelt zich in de gastheer te vermenigvuldigen en ziekten te verwekken.
   Dit proefschrift.
2. De mogelijkheid dat het door tabaksmozaïekvirus gecodeerde 30 kDa eiwit geen nieuwe plasmodesmata induceert maar het verlies van bestaande plasmodesmata voorkomt, wordt door Leonard en Zaitlin al te lichtvaardig terzijde geschoven.
   Leonard, D. and Zaitlin, M. (1982), Virology **117:** 416-424.
3. De door Reichman *et al.* gerapporteerde remming van de tabaksmozaïekvirus vermenigvuldiging na behandeling van bladponsjes met humaan interferon berust op toeval.
   Reichman, M., Devash, Y., Suhadolnik, R. J. and Sela, I. (1983), Virology **128:** 240-244.
   Antoniw, J., White, R. F. and Carr, J. P. (1984), Phytopath. Z. **109:** 367-371.
4. De immuniteit van het embryo die Hosokawa en Mori voorstellen als verklaring voor het niet met zaad overdraagbaar zijn van tabaksmozaïekvirus in *Petunia hybrida*, is niet uit de door hen gepresenteerde resultaten af te leiden.
   Hosokawa, D. and Mori, K. (1982), Ann. Phytopath. Soc. Japan **48:** 534-537.
5. De conclusie van Palmiter *et al.* dat de toegenomen groei van muizen die het "metallothionein-human growth hormone fusion gene" in hun chromosomen geïncorporeerd hebben, uitsluitend het gevolg is van het tot expressie komen van dit gen, is onvoldoende gefundeerd.
   Palmiter, R. D., Norstedt, G., Gelinas, R. E., Hammer, R. E. and Brinster, R. L. (1983), Science **222:** 809-814.
6. De bestudering van de "pathogenesis-related proteins" is van groter belang voor de plantenfysiologie dan voor de planteziektenkunde.
7. Planteziektenkundig onderzoek dient zich minder te richten op het pathogeen en diens eigenschappen, doch meer de (eigen)aardigheden van de gastheer in het onderzoek te betrekken.
8. De werkelijke "brain-drain" is niet het vertrek van academici van universiteiten naar bedrijfsleven, maar de huidige, grote werkloosheid onder afgestudeerde academici.
9. Er dienen betere mogelijkheden geschapen te worden om succesvolle onderzoeksprojecten sneller te kunnen continueren.

10. Gezien de eerste plaats die Nederlandse computerenthousiasten innemen bij het doorbreken van de beveiligingscodes van software, lijken berichten als zou Nederland een achterstand hebben op het gebied van informatica, overdreven.

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Wageningen, 6 februari 1985.

Aan mijn ouders
Voor Ans

Woord vooraf

Aan de totstandkoming van dit proefschrift hebben vele mensen op enigerlei wijze een bijdrage geleverd. Het is vrijwel ondoenlijk een ieder op deze plaats persoonlijk te danken maar toch wil ik hier graag enkele mensen met name noemen.

Allereerst mijn ouders voor de mogelijkheden die zij mij hebben gegeven om de door mij gekozen studie te volgen.

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De medewerkers van de fotografische dienst van de Binnenhaven, de heren Visser, Eimers en Brangert voor de uitstekende wijze waarop zij de soms zeer lastige eiwit patronen op gevoelige plaat wisten vast te leggen.

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De heer Seves van Grafisch bedrijf Ponsen & Looijen voor zijn hulp en uitleg bij het converteren van de op diskette opgeslagen tekst naar fotozetsel

Allex Haasdijk en Paul van Snippenburg voor het tekenen van de diverse figuren en hun hulp bij bij het monteren van posters en dit proefschrift.

Ook allen die hier niet genoemd konden worden, heel hartelijk bedankt.

CONTENTS

Chapter 1
General Introduction

1.1 Viral pathogenesis in plants

Plant virologists started their studies with the description of symptoms and the mode of virus transmission (Mayer, 1882). In the mid 1930s the first viruses were isolated and characterized. After the second world war the study of virus structure and of its replication *in vivo* and *in vitro* emerged. Meanwhile, there has been a growing interest in the study of host responses, partly because of the economic losses caused by virus diseases, partly because many of the symptoms developing in virus-diseased plants resemble or actually are perturbations in normal growth and development and, therefore, of high interest to plant physiologists, too.

The responses of plants to virus infection fall into two groups. On the one hand, a virus particle may succesfully invade a leaf parenchyma cell, but it may also be injected directly into the sieve tubes of the phloem by a vector (e.g., aphids). After penetration the virus may move through the whole plant causing a systemic infection and the plant may eventually develop symptoms. A selection of symptoms which may occur upon systemic infection is given in Table 1.

TABLE 1

Important Host Plant Responses to Systemic Virus Infection

Plant Part	Symptoms	Comments
Whole Plant	1. Reduction in size	Probably most common response
	2. Wilting	Dessication may lead to rapid death of plant
	3. Generalized necrosis	Leads to rapid death
Leaves	1. Vein clearing	Translucent tissue near veins
	2. Mosaic patterns	Very common response
	3. Blistering/distortion of lamina	Often associated with mosaic
	4. Enations	Abnormal leaflike growths from veins and midribs
	5. Epinasty	---
Flowers	1. "Breaking" of petal pigmentation	Often associated with mosaic in the leaves
	2. Malformation	---
	3. Necrosis	---

After: Matthews, 1980.

On the other hand, the virus may also remain localized at the site of penetration in local lesions that, for example, may consist of cells that have lost some chlorophyll (chlorotic lesions) or of a patch of dead cells (necrotic lesions). The latter type of reaction is called a hypersensitive reaction and gives the plant an effective 'field resistance' to a disease (Matthews, 1980). Which reaction occurs depends on the genetic constitution of both host and virus. Usually, infection of a given plant species with different viruses will cause different symptoms, but also one and the same virus may cause different symptoms on different plant species. A general feature of many plant viruses is that they spread systemically in one host but are localized in a different host. Conversely, plants allow systemic spread of one virus, while localizing another virus. Generally, virus localization is regulated by a small number of mendelian inhereting plant genes. This implies that each plant possesses the capacity both to localize infecting viruses and to respond with the development of systemic symptoms, and that viruses possess genetic information which enables them to either spread systemically or remain localized. Symptoms are therefore characteristic of the specific combination of a virus and its host.

One of the best studied host-virus combinations is tobacco (*Nicotiana tabacum* L.) infected with tobacco mosaic virus (TMV). All natural varieties of *N. tabacum* are sensitive to TMV (Holmes, 1960) and develop mosaic symptoms on the newly emerging leaves after infection of older leaves with the common (U1) strain of TMV. In contrast, *N. glutinosa* localizes TMV U1 at the site of penetration of the virus into the leaf, forming necrotic local lesions (Holmes, 1929). This hypersensitive reaction is controlled by a single dominant nuclear gene, designated *N*. By chromosome substitution (Gerstel, 1943, 1945), the *Hg* chromosome from *N. glutinosa* carrying the *N* gene has been incorporated into the *N. tabacum* cultivars Samsun, White Burley, and Xanthi, replacing the *H* chromosome and rendering the new cultivars Samsun NN

TABLE 2

Reaction of Tobacco Plants Posessing Different N-alleles upon Infection with Different TMV Strains

	TMV strain			
Host Allele	U1	U2	U8 (RMV)	1952 D/Ni 2338
N	R	R	R	R
N'	S	R	R	R
n^S	S	S	R	R
n (n')	S	S	S*	R

RMV: Holmes' ribgrass strain of TMV; R: resistant (hypersentitive reaction); S: sensitive (systemic mosaic); S*: sensitive (semi-systemic ring lesions).

(Holmes, 1938), White Burley NN (Valleau, 1952) and Xanthi-nc (Takahashi, 1956), respectively. These new cultivars respond to TMV U1 infection with the hypersensitive reaction of *N. glutinosa*.

However, there are several strains of TMV which do not spread systemically in host plants lacking the *N* gene (Table 2). In tobacco, four phenotypic alleles have been distinguished which regulate the interaction with TMV. These alleles can be distinguished by their reaction towards four different types of strains or mutants of the virus (Van Loon, 1972), as shown in Table 2.

From Table 2 it can be concluded that tobacco varieties lacking the *N*-gene, are perfectly capable of reacting hypersensitively. In fact, all tobacco varieties given in Table 2, respond hypersensitively to infection with tobacco necrosis virus (TNV). In all these cases where tobacco plants respond with a hypersensitive reaction, the reaction itself is characterized by essentially similar morphological, physiological and biochemical changes (Van Loon, 1982; Van Loon and Callow, 1983). Moreover, such changes can be induced not only by other viruses, but also by some fungi or bacteria and even certain chemicals provoke similar symptoms, so that the hypersensitive reaction is aspecific. Thus, the sensitivity of tobacco towards certain TMV strains is apparently not due to the absence of the resistance mechanism itself, but rather to the fact that under the influence of these strains the hypersensitive reaction is not elicited. Therefore, the host plant alleles involved in the specific interaction with the virus seem to have a regulatory function.

Mundry and Gierer (1958) observed that mutation with nitrous acid of TMV strain *vulgare* (U1 type) led to the loss of the ability to induce systemic mosaic symptoms in Java tobacco (which possesses the *N'* allele). Due to the fact that these mutants caused local lesions on Java tobacco, a mutation from the U1 to a U2-type strain may have occurred (cf. Table 2). Despite all attempts made, an artificial back mutation from U2 to U1 was never observed (Mundry, 1960). Kado and Knight (1966) isolated several mutants from wild type TMV strain U1 which produced local lesions in *N'* hosts. They defined the mutation site to lie at approximately 25% from the 3'-end of the TMV RNA (in their paper they concluded that it was 25% from the 5'-end, but Wilson *et al* (1976) showed that the RNA ends had been misidentified). Kado and Knight (1966) named the RNA in this region the 'local lesion gene'. Whereas the hypersensitive reaction itself represents an aspecific reaction and a viral function may be necessary to suppress the expression of the hypersensitive reaction, it is difficult to envisage a virus carrying information for this type of effect. Rather, the virus might have lost the ability to cause systemic disease. On these grounds, the following hypothesis can be proposed: Viruses capable of spreading systemically possess a function that enables them to evade localization by their host (Van Loon, 1980).

The existence of different strains and isolates of a virus capable of systemically infecting only host plant varieties with a specific genetic constitution, may then be explained by assuming that only in those specific combinations the viral function is ca-

pable of suppressing the localizing mechanism of the host. This implies that besides the functions needed for successful replication and synthesis of coat protein, the viral genome should contain information for systemic spread leading to different types of symptoms in different hosts. Working with nitrous acid-induced mutants of TMV *vulgare*, Mundry and Gierer (1958) distinguished as much as 8-12 different types of systemic mosaic symptoms in the combination tobacco-TMV, which they interpreted as resulting from a similar number of different genes. However, as we now know from the nucleotide sequence data (Goelet *et al*, 1982), the TMV genome is composed of three closely packed open reading frames in phase. These code for four polypeptides as shown in Fig. 1, the 183 kDa polypeptide being the readthrough

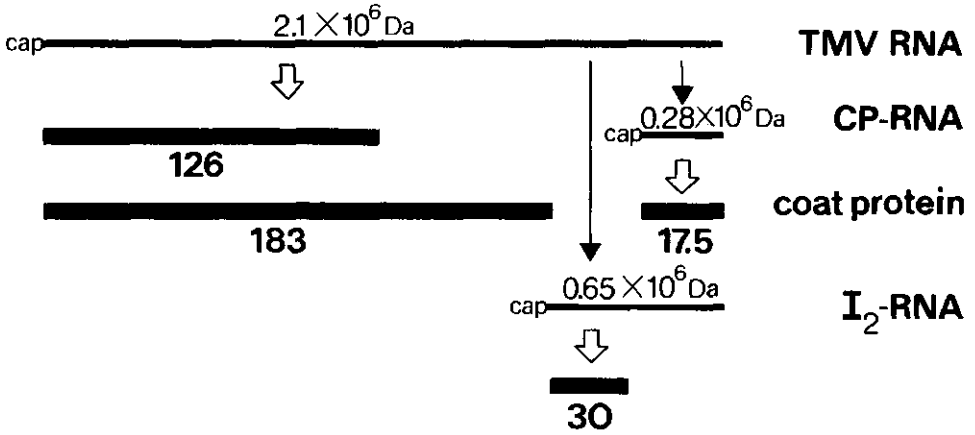

Fig. 1: Translation strategy of TMV (adapted from Beachy *et al*, 1976; Davies and Hull, 1982). Viral RNAs are indicated by thin lines, the translational products by solid bars. Closed and open arrows indicate presumed derivation of subgenomic RNAs and translational products, respectively. Numbers under solid bars refer to the molecular masses of the polypeptides as calculated by Goelet *et al*, 1982 (in kDa).

product of the 126 kDa gene. Out of phase with these three open reading frames are a number of potential start codons for genes coding for small proteins, but so far only the four translational products given in Fig. 1 have been detected in TMV-infected plants or protoplasts. These four polypeptides represent essentially all of the information content.

Only the function of the coat protein has been established with certainty. The function of the other translational products is not known yet. However, it has been suggested that the 30 kDa protein synthesized from TMV RNA *in vitro* (Leonard and Zaitlin, 1982) and also present in TMV-infected protoplasts (Ooshika *et al*, 1984), is involved in cell-to-cell transport (Leonard and Zaitlin, 1982); the high-mo-

lecular weight proteins may be virus-specific subunits of a RNA-dependent RNA polymerase (Scalla *et al*, 1978). Since these viral products are apparently needed and sufficient for successful multiplication and spread of the virus, it is to be expected that other plant positive-strand RNA viruses (including multicomponent viruses) possess functionally similar genetic information, as indeed seems to be the case for bromoviruses (for a review, see Davies and Hull, 1982). Some viruses may use a more complicated strategy to produce essentially similar types of polypeptides (e.g. the comoviruses (Davies and Hull,1982)), or additionally code for one or more proteins needed for arthropod transmission (see Zaitlin, 1983).

Since these products turn out to occupy essentially the entire coding capacity of the viral genome, there does not seem to be any information content left for specific symptom-inducing polypeptides. For instance, Fulton (1972, 1975) reported that in *Nicotiana* species infected with tobacco streak virus, certain strains of the virus induced profound dwarfing of the host, whereas others induced regularly dented leaves. It is hardly conceivable that the viral genome possesses genetic information for proteins involved in leaf morphogenesis. So, unless there is a marked pleiotropic effect of viral genes, the plant viral genome is too small to code for such a wide range of viral symptoms, for instance, for as many as 8-12 types of mosaic as reported by Mundry and Gierer (1958) for their nitrous acid-induced TMV mutants. However, viral constituents or translational products must be involved in the induction of disease and expression of symptoms (Zaitlin, 1979; Matthews, 1980, 1981).

In the absence of any specific pre-existing nutritional stress, it is unlikely that the actual sequestration of amino acids and nucleotides into virus particles has any direct connection with the induction of symptoms. Support for this view can be derived from observations that closely related strains of the same virus may multiply in a particular host to give a similar final concentration of virus and, yet, have different effects on host cell constituents and induce symptoms of greatly differing severity (Matthews, 1980). For instance, both TMV and barley yellow dwarf virus induce severe diseases but, nevertheless, yields of extractable virus are typically 5-10 mg and 50-150 ng per gram of tissue, respectively (Zaitlin, 1979). Moreover, viroids consist only of RNA and do not code for any known protein(s) but still cause severe symptoms. Finally, the fact that viral symptoms can be easily mutated, indicates that a general, non-specific sequestration mechanism is unlikely.

A primary role of the coat protein in the induction of disease symptoms seems improbable, too. This follows from the occurrence of mutants which have identical coat proteins but, nevertheless, induce very different symptoms. Moreover, in experiments with pseudo-recombinants of multi-partite viruses, it was found that generally symptom type does not segregate together with the coat protein gene (Habili and Francki, 1974). The role of non-structural viral proteins has likewise been suggested to be minimal. As yet no gene product responsible for initiating symptoms has been isolated or characterized. However, since plant viroids induce disease without being

translated, a possible involvement of the viral RNA itself in the induction of disease seems more likely. Matthews (1980) suggests that small segments of the viral genome might be replicated separately to act directly as repressors or derepressors of host functions.

1.2 Role of host genes and proteins

There are several indications that host genes are also involved in the initiation of disease symptoms. For instance, in non-infected plants certain chemicals can induce symptoms resembling the ones developing after certain virus infections (for a review, see Matthews, 1981). Toxins produced by arthropods as well as some insecticides may produce virus-like symptoms. Furthermore, treatments with plant growth regulators often produce effects mimicking virus disease. Already in 1953, Audus reported that the auxin 2,4-dichlorophenoxyacetic acid (2,4-D) can induce such growth abnormalities as shoestring appearance of leaves in tobacco, which bear resemblance to the effect of certain virus infections. Similar observations have been made in tomato and in grape (Matthews, 1981). Symptoms like stunting, epinasty, and malformations (Table 1) are similarly suggestive of disturbances of endogenous hormone metabolism. Pricking leaves of *N*-gene-containing tobacco plants with needles moistened with ethephon, that in the plant decomposes to yield ethylene, induces a reaction almost indistinguishable from the hypersensitive reaction developing after TMV inoculation (Van Loon, 1982). Furthermore, many of the changes occurring during this hypersensitive reaction are similar to those occurring in non-infected plants during artificial ageing due to detachment. Similarly, in plants developing mottling or mosaic symptoms, such as may be the case after infection of tobacco with cucumber mosaic virus (CMV), the loss of chlorophyll occurring in the light-green or yellow areas resembles ethylene-induced senescence. However, the development of mosaic symptoms upon TMV infection occurs only in newly emerging leaves; in leaves over approximately 15 mm in length at the time of inoculation no symptoms develop (Nilsson-Tillgren *et al*, 1969). Furthermore, Hirai and Wildman (1967) demonstrated that if actinomycin D, an inhibitor of DNA-dependent RNA synthesis, was introduced into small tobacco plants, the young leaves developed mosaic symptoms apparently similar to those induced by the common strain of TMV. These observations all indicate that symptoms and disease must arise from an interference of the infecting virus with host genes regulating normal growth and development.

If it is assumed that symptom induction requires the recognition of specific host functions involved in the regulation of plant growth and development, the molecular basis of sensitivity to viral infection may be sought at the level where genes regulating these processes are expressed, i.e. at the transcription of the DNA. Alterations in transcription will be reflected in (a) changes in mRNA populations (b) changes in the kind and quantity of the proteins having a regulatory role in specific gene expression, the chromatin-associated proteins. Since during the phase of exponential growth of a

tobacco plant approximately 27,000 different mRNAs are being transcribed in a developing leaf (Kamalay and Goldberg, 1980), looking for a small number of unknown mRNAs of a possibly rare class, is more or less looking for a needle in a haystack (cf. De Vries, 1983). Since the number of different chromatin-associated proteins is considerably less (Peterson and McConkey, 1976), it is simpler to investigate first whether there are changes in the constitution of the proteins associated with the chromatin.

Chromatin consists of a complex of DNA, RNA and protein. The chemical composition and structure of this complex is highly specific, and seems to be important both in packaging the large amounts of DNA found in eukaryotic nuclei and in the regulation of transcriptional activity (Thompson and Murray, 1981). The average chemical composition of tobacco chromatin is 1.0 DNA: 2.7 protein: 0.2 RNA (Gigot *et al*, 1976). The function of the RNA present in chromatin is not clear (Van Venrooij and Jansen, 1978). It has been postulated, but not proven, that this RNA has a function in gene regulation (Holmes *et al*, 1972). There are indications that RNA and proteins form a complex in the nucleus, which can regulate gene activity (LeStourgeon *et al*, 1977). RNA also functions as a primer in DNA replication.

The proteins present in the chromatin can be classified as histones and non-histones. The histones are small, very basic proteins characterized by high lysine and arginine contents. The function of the histones is mainly structural and lies primarily in their packaging of the DNA into nucleosomes (Thompson and Murray, 1981). Template accessibility may be modified by acetylation or phosphorylation, which loosen the histon-DNA interaction. However, so far regulatory functions for histones in specific gene expression have never been demonstrated. The histone composition does not change when pea seedlings elongate in response to treatment with gibberellic acid (Spiker and Chalkley, 1971), when RNA synthesis was inhibited in maize coleoptiles after treatment with abscisic acid (Bex, 1972), upon vernalization in wheat (Spiker and Krishnaswamy, 1973), or during the course of germination (Yoshida and Sasaki, 1977). Moreover, the histones of any given plant species are electrophoretically identical in different tissues (Pitel and Durzan, 1978a). Only histone H1 may vary and show quantitative differences in phosphorylation during the cell cycle (Stratton and Trewavas, 1981).

Non-histone chromatin proteins may also have a function in the maintenance of chromatin structure (Adolph *et al*, 1977; Paulson and Laemmli, 1977), but there are many indications that these proteins are responsible for regulating gene activity (Cartwright *et al*, 1982). The non-histones display at least three attributes required for specific gene regulatory elements. First, they are heterogeneous: HeLa cell non-histone proteins were resolved into at least 450 polypeptides (Peterson and McConkey, 1976). Second, in contrast to histones, the non-histones show an appreciable turnover during the cell cycle, and this rate may increase during cell proliferation (Garrard and Bonner, 1974). Third, non-histone proteins show tissue specificity. Tissue-specific differences in non-histone protein composition have been reported for various animal

tissues (Bekhor *et al*, 1974; Chiu *et al*, 1975; Kleinsmith, 1975) as well as for pea and pine seedlings (Lin *et al*, 1973; Mischke and Ward, 1975; Pitel and Durzan, 1978b). However, Towill and Noodén (1975) found the non-histone protein composition to be virtually identical in different tissues of maize seedlings. Changes in non-histone proteins were reported to be associated with pollen tube development (Pipkin and Larson, 1973), wheat embryo germination (Yoshida and Sasaki, 1977) and differentiation and dedifferentiation of tobacco callus tissue (Guerri *et al*, 1982).

An interesting feature of non-histone proteins is that they are subject to extensive phosphorylation (Kleinsmith, 1975; Trewavas, 1979). Phosphorylation of non-histones, like acetylation of histones, may weaken the interaction between the proteins and the DNA and, thereby, render the DNA accessible for transcription. As in animal systems, in plants phosphorylation of non-histone proteins is stimulated when transcriptional activity is enhanced (Trewavas, 1976; Murray and Key, 1978). During barley germination and upon abscisic acid treatment of *Lemna*, the patterns of non-histone protein phosphorylation change dramatically (Chapman *et al*, 1975). Nuclear protein phosphorylation *in vitro* is enhanced in nuclei isolated from 2,4-D-treated soybean hypocotyls compared to nuclei from untreated tissue. This change parallels 2,4-D-enhanced RNA polymerase activity of these nuclei and the *in vivo* levels of RNA synthesis (Murray and Key, 1978). Dunham and Yunghans (1977) reported that soybean chromatin reconstituted with phosphorylated non-histone proteins was a more efficient template for DNA replication than DNA reconstituted with unphosphorylated non-histone proteins.

However, it cannot be excluded that many of the alterations in non-histone phosphoproteins thought to be associated with gene regulation, are actually involved in post-transcriptional processing and/or packaging of RNA (Van Venrooij and Jansen, 1978). A major non-histone nuclear protein, known to decrease as *Physarum polycephalum* goes into a resting state, has been shown to be involved in RNA packaging (LeStourgeon *et al*, 1977). Changes in non-histone protein composition would then be the result of specific gene activity rather than its cause.

Particularly in animal systems, however, indications that specific regulatory elements of gene activity are present in the non-histone protein fraction are convincing. Many of these data have been obtained by chromatin reconstitution and transcription *in vitro*. Stein *et al* (1975) showed that the *in vitro* transcription of histone genes from HeLa cells was enhanced after addition of non-histone chromatin proteins from S-phase HeLa cells but not of non-histone proteins from G1-phase cells. Similarly, Gilmour and Paul (1975) demonstrated that the non-histone fraction of foetal liver chromatin but not of brain chromatin, specifically stimulated the expression of the globin genes in reconstituted chromatin from reticulocytes of mice. Finally, from chicken liver nuclei two DNA-binding proteins were isolated which inhibited the initiation of DNA transcription *in vitro* (Kiliansky *et al*, 1981). Yoshida *et al*. (1979) showed that the transcriptional activity of wheat chromatin was enhanced

upon addition of non-histone proteins isolated from seedlings, but was reduced with non-histone proteins from endosperm. Similarly, non-histone proteins from maize or pea plants that had been treated with gibberellic acid, stimulated the transcriptional activity of chromatin-associated RNA polymerase (Wielgat and Kleczkowski, 1981). Specific proteins regulating the expression of specific genes have not yet been identified in plants. However, it is to be expected that selective gene expression in plants is regulated in a similar fashion as in animal systems.

Although these observations indicate that non-histone proteins are involved in specific gene regulation, DNA-binding proteins that specifically regulate the transcription of defined genes have not yet been isolated. It is quite possible that these proteins constitute only a very small portion of the total non-histone protein fraction, making their detection and isolation extremely difficult. For instance, Lin and Riggs (1977) calculated that an eukaryotic equivalent to the bacterial *lac*-repressor would need to be present in no more than 15,000 copies per nucleus, even if all of the genome were accessible to repressor binding. If one postulates that much of the genome is masked, and therefore not available for binding, the amount of regulatory proteins needed will be proportionally lower.

This shows that detection of these proteins and their possible changes upon treatments altering specific gene expression will be difficult to assess using conventional separation and staining methods.

1.3 Approach

To overcome these difficulties, Thompson and Murray (1981) suggested two possible approaches. The first one is to identify non-histone proteins by virtue of their ability to interact with hormones or hormone-receptor complexes, thought to be involved in the activation of specific genes. The second one is to identify non-histone proteins that bind to specific DNA sequences, for instance by DNA-affinity chromatography. None of these approaches has as yet been applicable to our investigation whether alterations occur in the chromatin-associated protein composition upon infection with a virus. However, using two-dimensional gel electrophoresis, Peterson and McConkey (1976) were able to resolve at least 450 different polypeptides from HeLa cells. Because of their radioactive labeling during three days and the relatively fast turnover of proteins in dividing HeLa cells, the polypeptides synthesized *de novo* will be fairly representative of the non-histone chromatin proteins present. However, this situation does not apply to leaves.

To be able to not only detect newly synthesized polypeptides but also changes occurring in the existing ones, we aimed at isolating relatively high amounts of chromatin-associated proteins and using sensitive staining procedures. Furthermore, the chromatin isolation procedure of necessity resulted into a population of chromatin-associated proteins representating both the dark-green and the light-green areas from the mosaic-diseased leaves of TMV-infected plants, virus accumulating in the lighter

parts only. The methods developed to overcome these problems are described in Chapter 2. Experiments to investigate whether different viruses induce different changes, and whether these changes are specific to the virus or to the plant, are described in Chapter 3. In Chapter 4 the characteristics of a new 116 kDa protein, occurring specifically in TMV-infected tobacco plants, are described. Its close association with chromatin suggests that it might have a regulatory role. Proteolytic peptide mapping to compare this 116 kDa protein with a TMV-coded protein of similar molecular mass is described in Chapter 5. Finally, in Chapter 6 a modification of our method to prepare chromatin-associated proteins is presented which enables the study of protein changes by two-dimensional gel electrophoresis and silver staining, without the necessity of prior radioactive labeling of the plants.

References

Adolph, K. W., Cheng, S. M. and Laemmli, U. K. (1977). Role of nonhistone proteins in metaphase chromosome structure. *Cell* 12, 805-816.

Audus, L. J. (1953). Plant growth substances. Leonard Hill Ltd., London.

Beachy, R. N., Zaitlin, M., Bruening, G. and Israel, H. W. (1976). A genetic map for the cowpea strain of TMV. *Virology* 73, 498-507.

Bekhor, I., Anne, L., Kim, J., Lapeyre, J. N. and Stambauch, R. (1974). Organ discrimination through organ specific nonhistone chromosomal proteins. *Arch. Bioch. Biophys.* 161, 11-19.

Bex J. H. M. (1972). Effects of abscisic acid on nucleohistone and histone synthesis in maize coleoptiles. *Acta Bot. Neerl.* 21, 292-300.

Cartwright, I. L., Abmayr, S. M., Fleischmann, G., Lowenhaupt, K., Elgin, S. C. R., Keene, M. A. and Howard, G. C. (1982). Chromatin structure and gene activity: The role of nonhistone chromosomal proteins. In: *Critical Reviews in Biochemistry* 13, 1-86, CRC Press.

Chapman, K. S. R., Trewavas, A. J. and Van Loon, L. C. (1975). Regulation of the phosphorylation of chromatin-associated proteins in *Lemna* and *Hordeum*. *Plant Physiol.* 55, 293-296.

Chiu, J. F., Wang, S. Fujitani, H. and Hnilica, L. S. (1975). DNA-binding chromosomal nonhistone proteins. Isolation, characterization and tissue specificity. *Biochemistry* 14, 4552-4558.

Davies, J. W. and Hull, R. (1982). Genome expression of plant positive-strand RNA viruses. *J. gen. Virol.* 61, 1-14.

De Vries, S. (1983). Gene expression in developing pea seedlings. *PhD-thesis*, State University, Groningen.

Dunham, V. L. and Yunghans, W. N. (1977). Effects of nuclear proteins on the activity of soybean DNA polymerase. *Biochem. Biophys. Res. Commun.* 75, 987-994.

Fulton, R. W. (1972). Inheritance and recombination of strain-specific characters in tobacco streak virus. *Virology* 50, 810-820.

Fulton, R. W. (1975). The role of top particles in recombination of some characteristics of tobacco streak virus. *Virology* 67, 188-196.

Garrard, W. T. and Bonner, J. (1974). Changes in chromatin proteins during liver regeneration. *J. Biol. Chem.* 249, 5570-5579.

Gerstel, D. U. (1943). Inheritance in *Nicotiana tabacum*. XVII: Cytogenetical analysis of *glutinosa*-type resistance to mosaic disease. *Genetics* 28, 533-536.

Gerstel, D. U. (1945). Inheritance in *Nicotiana tabacum*. XIX: Identification of the *tabacum* chromosome replaced by one from *N.glutinosa* in mosaic-resistent Holmes' Samsun tobacco. *Genetics* 30, 448-454.

Gigot, C., Philips, G., Nicolaieff, A. and Hirth, L. (1976). Some properties of tobacco protoplast chromatin. *Nucleic Acid Res.* 3, 2315-2329.

Gilmour, R. S. and Paul, J. (1975). The *in vitro* transcription of the globin gene in chromatin. In: *Chromosomal proteins and their role in the regulation of gene expression*, pp 19-33. Ed. by G. S. Stein and L. J. Kleinsmith. Academic Press.

Goelet, P., Lomonossoff, G. P., Butler, P. J. G., Akam, M. E., Gait, M. J. and Karn, J. (1982). Nucleotide sequence of tobacco mosaic virus RNA. *Proc. Natl. Acad. Sci. USA* 79, 5818-5822.

Guerri, J., Culianez, F., Primo-Millo, E. and Primo-Yufera, E. (1982). Chromatin changes related to dedifferentiation and differentiation in tobacco tissue culture (*Nicotiana tabacum* L.). *Planta* 155, 273-280.

Habili, N. and Francki, R. I. B. (1974). Comparative studies on tomato aspermy and cucumber mosaic viruses. III. Further studies on relationship and construction of a virus from parts of two viral genomes. *Virology* 61, 443-449.

Hirai, A. and Wildman, S. G. (1967). Similarity in symptoms produced in tobacco plants by actinomycin D and TMV. *Virology* 31, 721-722.

Holmes, F.O. (1929). Local lesions in tobacco mosaic. *Bot.Gaz.* 87, 39-55.

Holmes, F.O. (1938). Inheritance of resistance to tobacco mosaic virus in tobacco. *Phytopathology* 28, 553-561.

Holmes, F.O. (1960). Inheritance in tobacco of an improved resistance to infection by tobacco mosaic virus. *Virology* 12, 59-67.

Holmes, D. S., Mayfield, J. E., Sander, G. and Bonner, J. (1972). Chromosomal RNA: Its properties. *Science* 177, 72-74.

Kado, C. I. and Knight, C. A. (1966). Location of a local lesion gene in tobacco mosaic virus RNA. *Proc. Natl. Acad. Sci. USA* 55, 1276-1283.

Kamalay, J. C. and Goldberg, R. B. (1980). Regulation of structural gene expression in tobacco. *Cell* 19, 935-946.

Kiliansky, Z., Hardy, K., Chiu, J. F. and Hnilica, L. S. (1981). Inhibition of DNA transcription by chromosomal nonhistone proteins. *Int. J. Biochem.* 13, 1127-1132.

Kleinsmith, L. J. (1975). Phosphorylation of non-histone proteins in the regulation of chromosome structure and function. *J. Cell. Physiol.* 85, 459-475.

Leonard, D. A. and Zaitlin, M. (1982). A temperature-sensitive strain of tobacco mosaic virus defective in cell-to-cell movement generates an altered viral-coded protein. *Virology* 117, 416-424.

LeStourgeon, W. M., Beyer, A. L., Christensen, M. E., Walker, B. W., Poupore, S. M. and Daniels, L. P. (1977). The packaging proteins of core hnRNP particles and the maintenance of proliferative cell states. *Cold Spring Harbor Symp. Quant. Biol.* 42, 885-898.

Lin, P. P. C., Wilson, R. F. and Bonner, J. (1973). Isolation and properties of nonhistone chromosomal proteins from pea chromatin. *Mol. Cell. Biochem.* 1, 197-207.

Lin, S. and Riggs, A. D. (1975). The general affinity of *lac* repressor for E.coli DNA: Implications for gene regulation in procaryotes and eucaryotes. *Cell* 4, 107-111.

Matthews, R. E. F. (1980). Host plant responses to virus infection. In: *Compr. Virol.* 16, 297-359. Ed. by H. Fraenkel-Conrat and R. R. Wagner. Plenum Press.

Matthews, R. E. F. (1981). In: *Plant Virology* 2nd Edition, Academic Press.

Mayer, A. (1882). Over de mozaikziekte van den tabak. Voorloopige mededeeling. *Tijdschr. Landbouwk. Groningen*, 359-364.

Mischke, B. G. and Ward, O. G. (1975). Isolation and tissue specificity of chromatin-associated proteins in *Vicia faba*. *Can. J. Biochem.* 53, 91-95.

Mundry, K. W. (1960). Mutationsuntersuchungen am Tabakmosaikvirus *in vitro*. I. Die Abhängigkeit des Erfolgs der Mutagenese mit HNO_2 von der Mutationsrichtung. *Z. Vererbungslehre* 91, 81-86.

Mundry, K. W. and Gierer, A. (1958) Die Erzeugung von Mutationen des Tabakmosaikvirus durch chemische Behandlung seiner Nucleinsäure *in vitro*. *Z. Vererbungslehre* 89, 614-630

Murray, M. G. and Key, J. L. (1978). 2,4-Dichlorophenoxyacetic acid-enhanced phosphorylation of soybean nuclear proteins. *Plant Physiol.* 61, 190-198.

Nilsson-Tillgren, T., Kolehmainen-Sevéus, L. and von Wettstein, D. (1969). Studies on the biosynthesis of TMV. I: A system approaching a synchronized virus synthesis in a tobacco leaf. *Mol. Gen. Genet.* 104, 124-141.

Ooshika, I., Watanabe, Y., Meshi, T., Okada, Y., Igano, K., Inouye, K. and Yoshida, N. (1984). Identification of the 30K protein of TMV by immunoprecipitation with antibodies directed against a synthetic peptide. *Virology* 132, 71-78.

Paulson, J. R. and Laemmli, U. K. (1977). The structure of histone-depleted metaphase chromosomes. *Cell* 12, 817-828.

Peterson, J. L. and McConkey, E. H. (1976). Non-histone chromosomal proteins from HeLa cells. A survey by high resolution, two dimensional electrophoresis. *J. Biol. Chem.* 251, 548-554.

Pipkin, J. L. and Larson, D. A. (1973). Changing patterns of nucleic acids, basic and acidic proteins in generative and vegetative nuclei during pollen germination and pollen tube growth in *Hippeastrum belladonna*. *Exp. Cell. Res.* 79, 28-42.

Pitel, J. A. and Durzan, D. J. (1978a). Chromosomal proteins of conifers. 1. Comparison of histones and nonhistone chromosomal proteins from dry seeds of conifers. *Can. J. Bot.* 56, 1915-1927.

Pitel, J. A. and Durzan, D. J. (1978b). Chromosomal proteins of conifers. 2. Tissue specificity of the chromosomal proteins of jack pine (*Pinus banksiana*). *Can. J. Bot.* 56, 1928-1931.

Scalla, R., Romaine, P., Asselin, A., Rigaud, J. and Zaitlin, M. (1978). An *in vivo* study of nonstructural polypeptides synthesized upon TMV infection and its identification with a polypeptide synthesized *in vitro* from TMV RNA. *Virology* 91, 182-193.

Spiker, S. (1975). An evolutionary comparison of plant histones. *Biochim. Biophys. Acta* 400, 461-467.

Spiker, S. and Chalkley, R. (1971). Electrophoretic analysis of histones from gibberellic acid-treated dwarf peas. *Plant Physiol.* 47, 342-345.

Spiker, S. and Krishnaswamy, L. (1973). Constancy of wheat histones during development. *Planta* 110, 71-76.

Stein, G., Stein, J., Thrall, C. and Park, W. (1975). Regulation of histone gene transcription during the cell cycle by nonhistone chromosomal proteins. In: *Chromosomal proteins and their role in the regulation of gene expression*, pp. 1-17. Ed. by G. S. Stein and L. J. Kleinsmith. Academic Press.

Stratton, B. R. and Trewavas, A. J. (1981). Phosphorylation of histone H1 during the cell cycle of artichoke. *Plant, Cell and Environment* 4, 419-426.

Takahashi, W. N. (1956). Increasing the sensitivity of the local-lesion method of virus assay. *Phytopathology* 46, 654-656.

Thompson, W. F and Murray, M. G. (1981). The nuclear genome: Structure and function. In: *The Biochemistry of Plants. A Comprehensive Treatise* 6, 1-81. Ed. by A. Marcus. Springer Verlag.

Towill, L. E. and Noodén, L. D. (1975). An electrophoretic analysis of the acid-insoluble chromosomal proteins from different organs of maize seedlings. *Plant Cell. Physiol.* 16, 1073-1084.

Trewavas, A. J. (1976). Post-translational modification of proteins by phophorylation. *Ann. Rev. Plant Physiol.* 26, 349-374.

Trewavas, A. J. (1979). Nuclear phosphoproteins in germinating cereal embryos and their relationship to the control of mRNA synthesis and the onset of cell division. In: *Recent advances in the biochemistry of cereals*, pp 175-208. Ed. by D. L. Laidman and R. C. Wyn Jones. Phytochemical Society Symposium 16.

Valleau, W.D. (1952). The evolution of susceptibility to tobacco mosaic virus in *Nicotiana* and the origin of the tobacco mosaic virus. *Phytopathology* 42, 40-42.

Van Loon, L.C. (1972). Pathogenese en symptoomexpressie in viruszieke tabak. Een onderzoek naar veranderingen in oplosbare eiwitten. *PhD-thesis*, Agricultural University, Wageningen.

Van Loon, L. C. (1980). Susceptibility to virus infection: A genetic-physiological problem. *Acta Bot.Neerl.* 29, 217.

Van Loon, L.C. (1982). Regulation of changes in proteins and enzymes associated with active defence against virus infections. In: *Active Defense Mechanisms in Plants*, pp 247-273. Ed. by R. K. S. Wood. Plenum Press.

Van Loon, L. C. and Callow, J. A. (1983). Transcription and translation in the diseased plant. In: *Biochemical Plant Pathology*, pp 385-414. Ed. by J. A. Callow. John Wiley & Sons.

Van Venrooij, W. and Janssen, D. B. (1978). HnRNP particles. *Mol. Biol. Rep.* 4, 3-8.

Wielgat, B. and Kleczkowski, K. (1981). Nonhistone chromosomal proteins from gibberellic acid treated maize and pea plants, and their effect on transcription *in vitro*. *Int. J. Biochem.* 13, 1201-1203.

Wilson, T. M. A., Perham, R. N., Finch, J. T. and Butler, P. J. G. (1976). Polarity of the RNA in tobacco mosaic virus particle and the direction of protein stripping in sodium dodecyl sulphate. *FEBS Lett.* 64, 285-289.

Yoshida, K. and Sasaki, K. (1977). Changes of template activity and proteins of chromatin during wheat germination. *Plant Physiol.* 59, 497-501.

Yoshida, K., Sugita, M. and Sasaki, K. (1979). Involvement of nonhistone chromosomal proteins in transcriptional activity of chromatin during wheat germination. *Plant Physiol.* 63, 1016-1021.

Zaitlin, M. (1979). How viruses and viroids induce disease. In: *Plant Disease, An Advanced Treatise* IV, 257-271. Ed. by J. G. Horsfall and E. B. Cowlings. Academic Press.

Zaitlin, M. (1983). Functions of the translation products of RNA plant viruses. *Plant Molecular Biology Reporter* 1-3, 111-115.

Chapter 2
Isolation and Electrophoretic Analysis of Chromatin-associated Proteins from Virus-infected Tobacco Leaves

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Summary

Nuclei from young tobacco leaves were isolated by tissue homogenization in an Omnimixer and repeated grinding in a Potter homogenizer. With this method up to 45 % of total leaf DNA was released. Examination of the nuclei by interference-contrast microscopy showed them to be intact. Chromatin prepared from these nuclei contained DNA, RNA, and protein in a ratio of 1 : 0.05 : 2.8. A slightly lower yield of nuclei was obtained from young, mosaic-diseased leaves from plants infected with tobacco mosaic virus (TMV), but infection did not affect the overall compositional characteristics of the chromatin.

Analysis of the chromatin proteins in polyacrylamide gels containing SDS showed that the only consistent alteration upon TMV infection was the induction of an apparently new protein of *c.* 116,000 D. This change was not observed upon electrophoresis in an acidic urea system. Thus, whereas TMV multiplies in the cytoplasm, upon infection a discrete alteration in the nuclear chromatin-associated proteins is induced which may be related to the expression of the characteristic mosaic symptoms.

Key words: Nicotiana tabacum, tobacco mosaic virus, chromatin proteins.

Introduction

Mosaic symptoms are a characteristic feature of many virus diseases. In tobacco, tobacco mosaic virus (TMV) induces a characteristic light green-dark green pattern on the young developing leaves. The light green areas abound with virus particles and physiologically and biochemically resemble senescent leaves. On the contrary, the dark green areas are almost devoid of virus and remain physiologically young (Matthews, 1981).

The accelerated ageing of light green areas does not result from exhaustion of the host due to its being forced to synthesize additional nucleic acid and protein for viral replication. The «masked» strain of TMV replicates almost to the same extent as the common strain without provoking symptoms. Furthermore, after infection with the common strain, host protein synthesis is only transiently inhibited (Fraser and Gerwitz, 1980). Symptoms are therefore likely to result from interference with the

Abbreviations: EDTA = ethylene diamino tetra acetic acid; SDS = sodium dodecyl sulphate; TMV = tobacco mosaic virus.

regulation of development, the common strain blocking chlorophyll synthesis and inducing premature ageing in some of the cells of the young developing leaves resulting in local chlorosis (Zaitlin, 1979).

Differential gene expression regulating plant growth and development occurs in the nucleus, whereas viral RNA and protein synthesis take place in the cytoplasm. If host metabolism is perturbed at the level of the genome, virus-induced alterations must be passed on to the nucleus and influence the transcription of DNA into RNA.

Particularly the non-histone chromatin proteins are considered to be the regulators of specific gene expression (Trewavas, 1979). Therefore we looked for changes in the chromatin-associated proteins to determine as to how far the induction of mosaic symptoms in tobacco leaves may be related to alterations in genetically-determined leaf development.

Materials and Methods

Plants and virus. Tobacco plants (*Nicotiana tabacum* L. cv. Samsun) were grown in a greenhouse at a minimum temperature of 20 °C and 50–80 % relative humidity. From October till March additional illumination was provided by Philips HPI/T 400 W lamps, ensuring a minimum light intensity of 10,000 Lux for $16\,h \cdot day^{-1}$.

When 7 to 8 weeks old, plants were inoculated on the youngest fully-grown leaf with purified TMV W U1 ($10\,\mu g\,ml^{-1}$). Control plants were similarly inoculated with distilled water.

Young, expanding leaves, 4–8 cm in length, were harvested 8–10 days later, when systemic mosaic symptoms became evident.

Isolation of nuclei. To ensure recovery of a representative sample of nuclei, high yields were desired. To this end several methods were compared and a modification of the methods of Hamilton et al. (1972) and Van Loon et al. (1975) was finally adopted.

Leaves were harvested around 8.45 a.m. and stored on ice. Midribs were removed and nuclei were isolated as outlined in Figure 1. All steps were carried out at 0°–4 °C.

Alternatively, nuclei were obtained by lysis of protoplasts. Protoplasts were prepared and nuclei isolated as described by Gigot et al. (1976).

Intactness of the nuclei was checked by interference contrast microscopy. The washed nuclear pellet still contained starch grains and crystals. Attempts to remove these contaminants by centrifugation through a sucrose cushion (Hamilton et al., 1972) or colloidal silica gradients (Luthe and Quatrano, 1980) were unsuccessful and resulted in substantial losses of nuclei. Therefore, the white-greyish nuclear pellet obtained after the second low-speed centrifugation was used immediately for chromatin isolation.

Preparation of chromatin from nuclei. Nuclei were lysed by resuspending the washed nuclear pellet in 25 ml 50 mM Tris-HCl, 1 mM EDTA, 10 mM β-mercaptoethanol, 1 mM $NaHSO_3$, pH 8.0, in an all-glass Potter homogenizer. The homogenate was centrifuged for 10 min at 10,000 *g*. The crude chromatin pellet was washed at least three times with 50 mM Tris-HCl, 10 mM β-mercaptoethanol, 1 mM $NaHSO_3$, pH 8.0. Finally the chromatin, after resuspension in 4–5 ml of washing buffer, was layered over 19 ml 10 mM Tris-HCl, 1.7 M sucrose, 10 mM β-mercaptoethanol, 1 mM $NaHSO_3$, pH 8.0. The upper one-third of the tube was stirred to form a crude gradient and the contents were spun at 50,000 *g* for 3 h. The resulting pellet, still containing some starch grains, was used as the purified chromatin.

Isolation of chromatin-associated proteins. Chromatin-associated proteins were dissociated from the DNA by resuspending the purified chromatin in 1.0 ml 10 mM Tris-HCl, 3 M NaCl, 6 M urea, 1 mM $NaHSO_3$, 1 % β-mercaptoethanol, pH 8.0, after Shaw and Huang (1970). DNA

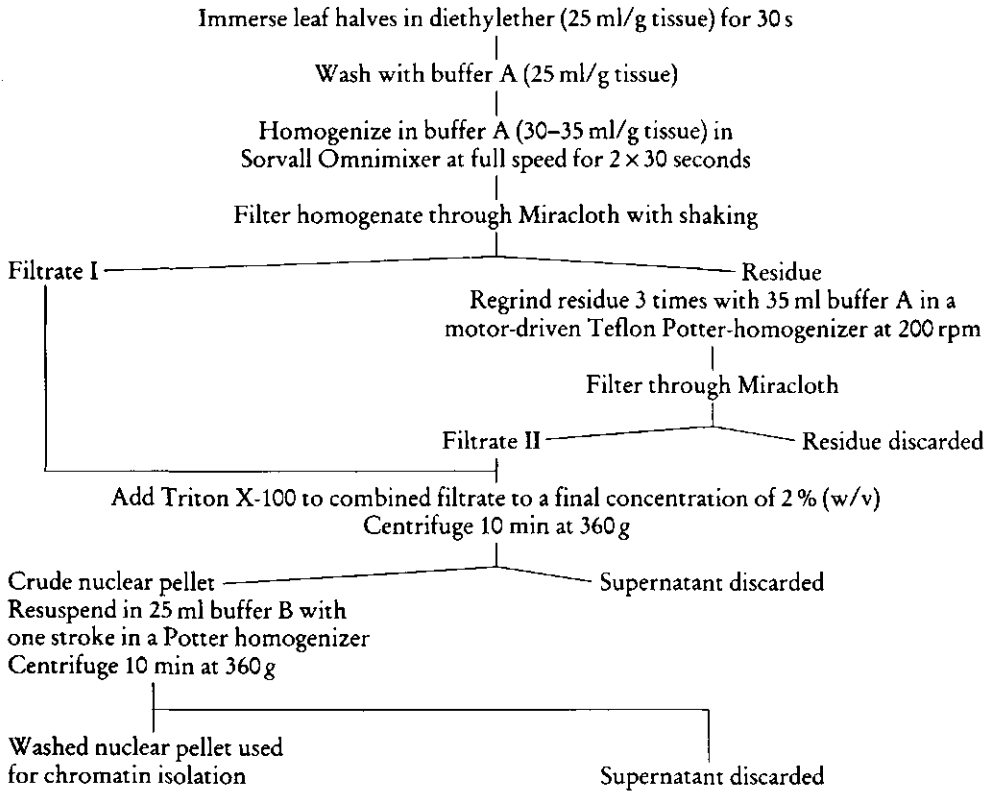


Fig. 1: Scheme for the isolation of nuclei from tobacco leaves.
Buffer A: 50 mM Tris-HCl, 0.3 M sucrose, 5 mM $MgCl_2$, 70 mM β-mercaptoethanol, pH 7.6;
Buffer B: 50 mM Tris-HCl, 0.3 M sucrose, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol, pH 7.6.

was removed by centrifugation at 100,000 *g* for 18 h. The protein-containing supernatant was used for the electrophoretic analysis. To distinguish between acid-soluble and acid-insoluble proteins, chromatin samples were extracted with 0.4 N H_2SO_4 according to Towill and Noodén (1973). Acid-soluble histones were separated from the acid-insoluble non-histone proteins by low-speed centrifugation.

Analysis of chromatin-associated proteins. Chromatin-associated proteins were analyzed by electrophoresis either on the basis of size, in 7.5 % and 10 % polyacrylamide gels containing 0.1 % SDS (Laemmli, 1970), or on the basis of both charge and size in 15 % acidic urea gels (Panyim and Chalkley, 1969). For the SDS system protein samples were dialyzed first against

10 mM Tris-HCl, 6 M urea, 1 mM $NaHSO_3$, 1 % SDS, 1 % β-mercaptoethanol, pH 8.0 for 4 h at room temperature, then against 10 mM Tris-HCl, 1 mM $NaHSO_3$, 0.1 % SDS, 0.1 % β-mercaptoethanol, pH 8.0 for 18 h at 4 °C. No differences were observed when samples were heated for 1 min in a boiling waterbath or not. For the acidic urea system, samples were dialyzed against 0.9 N acetic acid, 6 M urea, 1 mM $NaHSO_3$, 1 % β-mercaptoethanol.

Electrophoresis in discontinuous SDS-gels was conducted at 12°–15 °C; 60 V was applied until the bromophenol blue marker reached the separation gel and then electrophoresis was continued at 150 V until the marker had migrated within a few mm from the bottom of the gel.

Acidic urea gels were pre-electrophoresed for 1 h at 80 V. After sample application the gels were run for 22 h at 110 V at 2°–4 °C.

Initially gels were stained with 0.25 % Coomassie Brilliant Blue in methanol : acetic acid : water (5 : 1 : 4, v/v) and destained in methanol : acetic acid : water (5 : 7 : 88, v/v) at room temperature. Because of the rather low sensitivity of this stain, the silver stain of Morissey (1981) was also used.

Determination of DNA, RNA, and protein. Total leaf DNA was isolated according to Hamilton et al. (1972). DNA was determined with the diphenylamine method of Burton (1956) with calf thymus DNA as a standard.

Nucleic acids present during chromatin isolation and purification were determined using the method described by Hutchinson and Munro (1961). Samples taken at different steps during the isolation procedure were mixed with 5 vol. of ethanol at –20 °C. The precipitate was collected by centrifugation for 10 min. at 10,000 *g* and washed successively with ethanol and 0.25 M $HClO_4$.

RNA was hydrolyzed after resuspension of the pellet in 2.5 ml 0.3 N KOH at 37 °C for 18 h. After acidification, chilling and centrifugation of the sample, the supernatant was used for RNA determination, with the orcinol method of Ceriotti (1955), using yeast RNA as a standard.

The DNA-containing precipitate was resuspended in 2.0 ml 0.5 N $HClO_4$ and DNA was solubilized by incubation at 70 °C for 20 min.

Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Analysis of nucleic acids. Total leaf nucleic acid was extracted by the method of Laulhere and Rozier (1976). Nucleic acids at the different steps of chromatin isolation were extracted with the SDS-phenol method of Kirby (1965). After precipitation with 2.5 Vol. of 96 % ethanol at –20 °C for 24–48 h, nucleic acids were taken up in 100 mM Tris-acetate, 50 mM sodium acetate, 5 mM EDTA, 0.1 % SDS, pH 7.8, and analyzed in 2.7 % polyacrylamide gels according to Loening (1967). Gels were stained for 30 min with ethidiumbromide (1 $mg \cdot l^{-1}$) and examined under UV light.

Results

Isolation of nuclei. After TMV infection, mosaic symptoms become apparent only in young, developing tobacco leaves. Therefore, leaves were harvested when 4–8 cm long. At this stage, cell division has been completed and rapid elongation ensues.

The average amount of DNA present in these leaves was 1.05 ± 0.31 $mg \cdot g^{-1}$ fresh wt, variations being related to the season. This value is slightly lower than that reported by Hamilton et al. (1972) for similarly young leaves from the tobacco cultivar White Burley.

Upon homogenization of the tissue in an Omnimixer according to Hamilton et al. (1972) the average amount of DNA released was 17–19 %, most of the nuclei remaining trapped in the sheared cell wall network.

Further purification through sucrose solutions resulted in final yields of pure nuclei of 4–5 % of the total leaf DNA. Purification on colloidal silica gradients gave equally poor yields. Moreover, observation of these nuclei under the microscope showed them to be mostly disrupted.

To circumvent the loss of nuclei in the cell wall fraction, isolation from protoplasts was attempted. However, removal of the epidermis from the young fragile leaves was

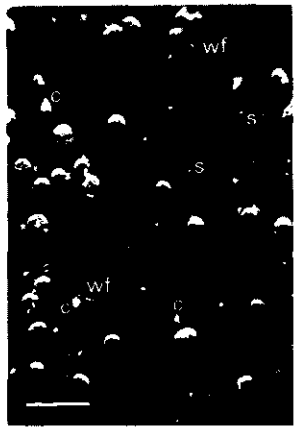


Fig. 2: Interference-contrast micrograph of once-washed tobacco leaf nuclei. Magnification 440×. Bar represents 10 μm. Impurities consist of starch grains (s), crystals (c), and some cell wall fragments (wf).

virtually impossible and protoplast yields were correspondingly low. Since only about 6 % of the nuclei were recovered by first isolating protoplasts, this procedure was inferior to the direct isolation method of Hamilton et al. (1972). This latter procedure was then further improved by adapting the method of Van Loon et al. (1975).

By homogenizing in a large volume of buffer and repeated grinding of the residue in a Potter homogenizer, many nuclei were freed from the cell debris. With this method up to 45 % of the total leaf DNA was released during homogenization. The nuclei present in the washed nuclear pellet were intact when examined by interference-contrast microscopy (Fig. 2).

Since extensive purification resulted in loss of 60–75 % of the nuclei, while most of

the visible contamination such as starch grains and crystals was still detectable, nuclei washed one time were lysed and the resulting chromatin was further purified as outlined in Materials and Methods.

Chromatin purification. Purification was checked by following the nucleic acid composition in the different fractions during isolation and purification (Fig. 3). Upon polyacrylamide gel electrophoresis of total nucleic acids from either healthy or TMV-infected leaves, a major slow-moving band was seen as well as bands of cytoplasmic and chloroplast ribosomal RNAs. The latter bands were absent if samples were treated with ribonuclease prior to electrophoresis, confirming their identity as plant-ribosomal RNAs. Chloroplast 23S RNA was essentially lacking in preparations from TMV-infected leaves, a phenomenon typical of senescing leaves (Leaver and Ingle, 1971). The RNA bands were only faint in the nuclear pellet (lane 4) and absent from the purified chromatin fraction (lane 6), indicating adequate removal of cytoplasmic

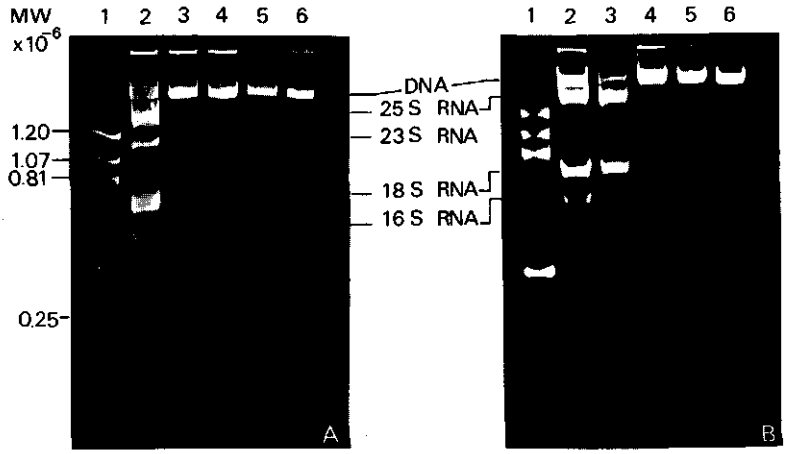


Fig. 3: Electrophoretic patterns in 2.7% polyacrylamide gels of nucleic acids from (A) non-infected and (B) TMV-infected tobacco leaves at the different isolation steps. Lane 1: Cowpea chlorotic mottle virus RNAs used as markers; 2: Total leaf nucleic acid; 3: Combined homogenate before addition of Triton X100; 4: Washed nuclei; 5: Crude chromatin from lysed nuclei; 6: Purified chromatin. Each lane contains the equivalent of 1 μg of total leaf nucleic acid.

contaminants. Viral RNA was not resolved on the gel. However, whereas fractions up to the purified nuclei proved infectious to *Nicotiana glutinosa*, no infectivity was found in the undiluted chromatin fraction.

The major, slow-moving band present in all samples was resistant to ribonuclease and sensitive to desoxyribonuclease, stained strongly with methyl green, and thus

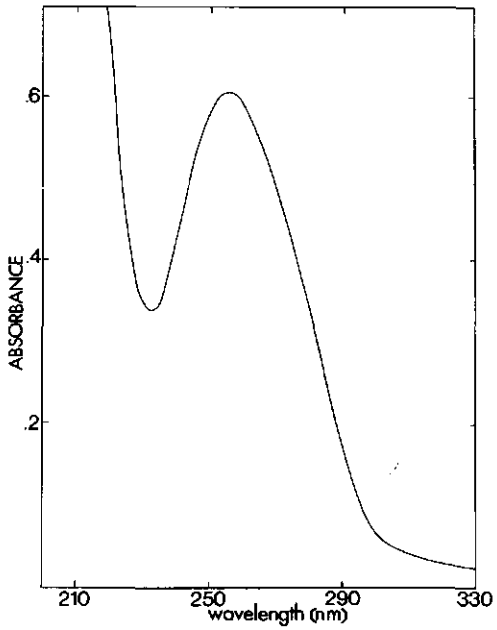


Fig. 4: UV absorbance spectrum of chromatin in 0.15 M NaCl, 0.015 M Na-citrate, pH 7.5.

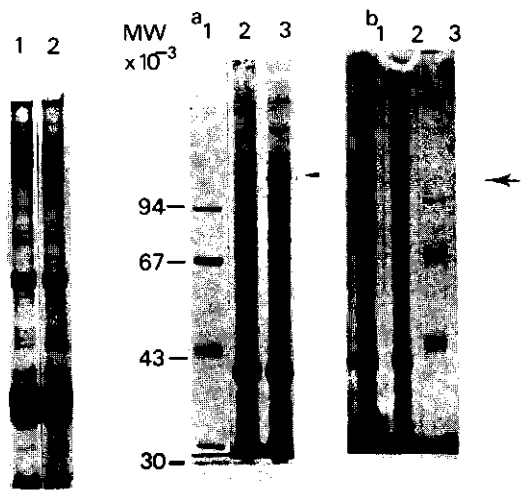
constitutes DNA. The decrease in intensity of this band reflects the gradual loss of some of the DNA during chromatin purification.

After purification the chromatin showed a characteristic UV spectrum with a maximum at 260 nm and a spectral ratio A_{260} : A_{280} of 1 : 0.59 (Fig. 4). The purified chromatin contained DNA, RNA and protein in a mean ratio of 1 : 0.05 : 2.8, while the mean ratio of DNA : acid-soluble : acid-insoluble protein was 1 : 0.9 : 1.7. This is well in accordance with the properties of tobacco leaf chromatin prepared from protoplasts by Gigot et al. (1976).

The yield of nuclei from TMV-infected leaves was always around 40 %, slightly lower than that from non-infected leaves. However, TMV infection had no influence on the overall compositional characteristics of the chromatin.

Analysis of chromatin-associated proteins. Chromatin-associated proteins were separated in up to 50 components when subjected to electrophoresis in either polyacrylamide acidic urea gels, or in 7.5 % or 10 % polyacrylamide gels containing 0.1 % SDS (Figs. 5–7).

In both electrophoretic systems the major, rapidly migrating protein bands constituted the acid-soluble histones, whereas the acid-insoluble non-histone proteins were present as bands of lower intensity throughout the entire gel. Infection with



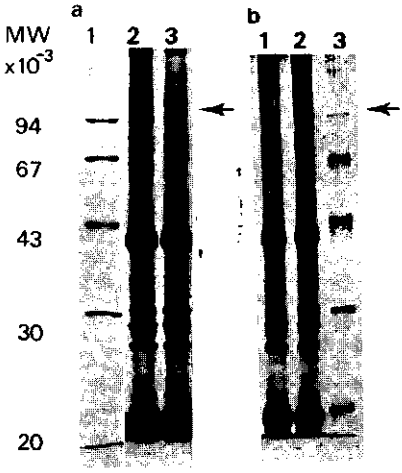


Fig. 5 Fig. 6 Fig. 7

Fig. 5: Electrophoretic patterns in acidic urea 15 % polyacrylamide gels of chromatin proteins, stained with Coomassie Blue. Lane 1: 16 μg total chromatin proteins from non-infected leaves; 2: 18 μg from TMV-infected leaves.

Fig. 6: Electrophoretic patterns in 7.5 % polyacrylamide gels, containing SDS, of chromatin proteins, stained with (A) Coomassie Blue, and (B) silver. (A) Lane 1: Marker proteins: phosphorylase b (94,000 D), bovine serum albumin (67,000 D), ovalbumin (43,000 D) and carbonic anhydrase (30,000 D); 2: 18 μg total chromatin proteins from non-infected leaves; 3: 18 μg from TMV-infected leaves. (B) Lane 1: 1.8 μg total chromatin proteins from non-infrected leaves; 2: 1.8 μg from TMV-infected leaves; 3: Marker proteins.

Fig. 7: Electrophoretic patterns in 10 % polyacrylamide gels containing SDS of chromatin proteins, stained with (A) Coomassie Blue, and (B) silver. Samples as in Fig. 6, except that the marker proteins also contained trypsin inhibitor (20,000 D).

TMV did not significantly alter the pattern obtained with the acidic urea system (Fig. 5). In contrast, upon SDS electrophoresis one major difference between non-infected and TMV-infected leaves could be discerned (Figs. 6, 7). Upon TMV infection, an apparently new, relatively strong band became evident, corresponding to a MW of 116,000 D. This band was best resolved in gels containing 7.5 % polyacrylamide (Fig. 6). No further consistent alterations were evident in either 7.5 % or 10 % gels and upon either Coomassie Blue or silver staining. Thus, infection with TMV caused a single discrete alteration in the pattern of non-histone chromatin-associated proteins in tobacco leaves.

Discussion

By improving existing techniques, notably by homogenization in large volumes of buffer, it was possible to isolate up to 45% of the nuclei present in young tobacco leaves. Such large yields are required to ensure recovery of a representative sample of nuclei. This is all the more required, because the mosaic pattern is composed of alternating light green and dark green spots. So far, we cannot say whether the nuclei isolated from infected plants were a representative sample from both areas or were predominantly derived from either the light green or the dark green tissue. However, since the yield of nuclei from TMV-infected leaves was only slightly lower than that from healthy leaves, a selective extraction seems rather unlikely.

Although the overall compositional characteristics were similar for chromatin prepared from non-infected and from TMV-infected leaves, one major alteration in the chromatin-associated proteins was evident upon SDS gel electrophoresis. This change was not revealed in the highly acidic urea system, underlining the importance of the use of more than one electrophoresis system to study alterations in complex mixtures of proteins. It can be anticipated that two-dimensional electrophoresis will reveal further differences and this technique will be adopted in future experiments.

Since TMV multiplies in the cytoplasm, the observed change in the pattern of chromatin-associated proteins is likely to be the result of reactions of the host plant to infection. This change may be related to altered gene expression regulating either premature leaf ageing in light green areas or virus exclusion from dark green areas, or both. Comparison of chromatin protein profiles in leaves of different developmental stages, and infected with viruses causing different types of symptoms, may shed further light on the significance of the observed alteration.

Acknowledgements

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References

Burton, K.: A study of the conditions and mechanisms of the diphenylamine reaction for the colorimetric estimation of desoxyribonucleic acid. Biochem. J. *62*, 315–323 (1965).

Ceriotti, G.: Determination of nucleic acids in animal tissues. J. Biol. Chem. *214*, 59–70 (1955).

Fraser, R. S. S. and A. Gerwitz: Tobacco mosaic virus infection does not alter the polyadenylated messenger RNA content of tobacco leaves. J. Gen. Virol. *46*, 139–148 (1980).

Gigot, C., G. Phillips, A. Nicolaieff, and L. Hirth: Some properties of tobacco protoplast chromatin. Nucleic Acid Res. *3*, 2315–2329 (1976).

Hamilton, R. H., U. Kunsch, and A. Temperli: Simple rapid procedures for the isolation of tobacco leaf nuclei. Anal. Biochem. *49*, 48–57 (1972).

HUTCHINSON, W. C. and H. N. MUNRO: The determination of nucleic acids in biological materials. A review. The Analyst *86*, 768–810 (1961).

KIRBY, K. S.: Isolation and characterization of ribosomal nucleic acid. Biochem. J. *96*, 266–269 (1965).

LAEMMLI, U. K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature *227*, 680–685 (1970).

LAULHERE, J.-P. and C. ROZIER: One-step extraction of plant nucleic acids. Plant Sci. Letters *6*, 237–242 (1976).

LEAVER, C. J. and J. INGLE: The molecular integrity of chloroplast ribosomal ribonucleic acid. Biochem. J. *123*, 235–243 (1971).

LOENING, U. E.: The fractionation of high molecular weight ribonucleic acids by polyacrylamide gel electrophoresis. Biochem. J. *102*, 251–257 (1967).

LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL: Protein measurement with the Folin phenol reagent. J. Biol. Chem. *193*, 265–275 (1951).

LUTHE, D. S. and R. S. QUATRANO: Transcription in isolated wheat nuclei I. Isolation of nuclei and elimination of endogenous ribonuclease activity. Plant Physiol. *65*, 305–308 (1980).

MATTHEWS, R. E. F.: Plant Virology, 2nd Ed., 190–209, Academic Press, New York and London, 1981.

MORRISSEY, J. H.: Silver stain for proteins in polyacrylamide gels. A modified procedure with enhanced uniform sensitivity. Anal. Biochem. *117*, 307–310 (1981).

PANYIM, S. and R. CHALKLEY: High resolution acrylamide gel electrophoresis of histones. Arch. Biochem. Biophys. *130*, 337–346 (1969).

SHAW, L. M. J. and R. C. C. HUANG: A description of two procedures which avoid the use of extreme pH conditions for the resolution of components isolated from chromatins prepared from pig cerebellar and pituitary nuclei. Biochemistry *9*, 4530–4542 (1970).

TOWILL, L. E. and L. D. NOODÉN: An electrophoretic analysis of the acid-insoluble chromosomal proteins from different organs of maize seedlings. Plant & Cell Physiol. *14*, 851–863 (1973).

TREWAVAS, A.: Nuclear phosphoproteins in germinating cereal embryos and their relationship to the control of mRNA synthesis. In: Recent advances in the biochemistry of cereals, 175–208, D. L. LAIDMAN and R. C. WYN JONES, (Eds.), Phytochemical Society Symposium 16, 1979.

VAN LOON, L. C., A. TREWAVAS, and K. S. R. CHAPMAN: Phosphorylation of chromatin-associated proteins in *Lemna* and *Hordeum*. Plant Physiol. *55*, 288–292 (1975).

ZAITLIN, M.: How viruses and viroids induce disease. In: Plant disease, an advanced treatise, IV: How pathogens induce disease, 257–271, J. G. HORSFALL and E. B. COWLING (Eds.). Academic Press, New York, 1979.

Chapter 3
Evidence for an Association of Viral Coat Protein with Host Chromatin in Mosaic-Diseased Tobacco Leaves

Summary

The effect of infection with different viruses on the chromatin-associated protein constitution of young, developing tobacco leaves was investigated using SDS and 2-D polyacrylamide gel electrophoresis. When systemic mosaic symptoms developed, discrete alterations in the protein profile were evident. The changes observed upon tobacco mosaic virus (TMV) infection consisted of the appearance of two additional protein bands with molecular mass of approximately 116 kDa and 20 kDa, respectively. Upon infection with cucumber mosaic virus (CMV), a 'green' isolate induced a single protein of about 28 kDa, whereas the 'yellow' strain P6 induced a similar protein of about 29 kDa. The 20 kDa TMV-induced protein was identified serologically as the viral coat protein, and the two CMV-induced proteins co-migrated with the coat proteins of the respective CMV strains. Virus infections resulting in necrosis, whether localized in the inoculated leaves or followed by systemic spread, did not induce any changes. Neither did treatment with 1-aminocyclopropane-1-carboxylic acid (ACC), to induce premature senescence in young, developing leaves, or natural senescence of old leaves on flowering plants induce any changes comparable to TMV infection. Since purified TMV coat protein did not associate with chromatin upon incubation *in vitro* with nuclei from non-infected tobacco plants, the interaction of viral coat protein with host chromatin is considered to be specific. The possibility that this association plays a role in symptom expression is discussed.

Introduction

The mosaic appearing in the young, developing leaves of tobacco plants upon infection with tobacco mosaic virus (TMV) is composed of alternating light green and dark green areas. The dark green 'islands' are almost devoid of virus and remain physiologically young, whereas the light green areas abound with virus and biochemically and physiologically resemble senescent leaves (11). Leaves larger than about 2 cm in length at the time of inoculation do not develop mosaic symptoms (11, 16), although high amounts of virus are synthesized in these leaves. This indicates that virus multiplication does not necessarily lead to symptom formation, as evidenced also by the existence of a 'masked' strain of TMV which multiplies to substantial levels in tobacco but does not induce visible symptoms (7). Since different viruses which multiply to low and high concentration can both produce severe symptoms, there is no general relationship between the extent of virus multiplication and the severity of the symptoms produced (30). Thus, symptoms do not necessarily result from exhaustion of the host due to its being forced to synthesize additional nucleic acid and protein. Rather, the sensitivity of exclusively the newly developing leaves to the induc-

tion of mosaic symptoms suggests that these symptoms arise due to an interference with normal leaf development.

The regulation of plant growth and development depends on differential gene expression, which is considered to be, in turn, regulated by the chromatin-associated non-histone proteins (23, 24). In a previous paper (27) it was shown that chromatin isolated from young, mosaic-diseased leaves of TMV-infected Samsun tobacco plants, contained an apparently new protein with a molecular mass of about 116 kDa. Since TMV multiplies in the cytoplasm (11), the observed change in the chromatin-associated protein constitution is unlikely to be related to viral replication. Instead, it might well be involved in the expression of symptoms.

The main feature of the light green areas in mosaic-diseased leaves is abnormal chloroplast development and premature chlorophyll loss (11), the latter being a characteristic of senescing leaves. Leaf senescence in tobacco is enhanced by ethylene (1). Hence, if the occurrence of the 116 kDa protein among the chromatin-associated proteins after TMV infection is related to the premature leaf senescence of the light green areas, one expects that artificial ageing of the leaves by treatment with 1-aminocyclopropane-1-carboxylic acid (ACC), the natural precursor of ethylene, will induce a similar change. If, on the other hand, the change is not related to senescence but connected with the presence of an infecting agent, different viruses causing similar symptoms might induce either the same, or different changes in the protein profile. To investigate the specificity of the observed change in TMV-infected Samsun tobacco, different tobacco cultivars were infected with viruses causing different types of symptoms, and their chromatin-associated protein constitution was analysed and compared with that of senescing and ageing leaves.

Materials and methods

Plants

Tobacco plants (*Nicotiana tabacum* L.) cvs Samsun, Samsun NN and White Burley were grown in a greenhouse as described previously (27). For high-temperature treatments, plants were placed in a growth cabinet (31-32 °C, 60-80% RH, 16 h photoperiod of fluorescent light at an intensity of 7.5 $W \cdot m^{-2}$).

When 7-8 weeks old, plants were inoculated by gently rubbing two fully-grown, carborundum-dusted leaves with a foam plastic pad dipped in virus suspension, followed by rinsing with tap water. Control plants were similarly treated with buffer solution.

To induce premature senescence (artificial ageing), young developing leaves of Samsun and Samsun NN tobacco (4-8 cm in length) were detached and their midribs removed. One half of each leaf was vacuum-infiltrated with a 0.1 mM solution of ACC; the other half was infiltrated with water and served as a control. After allowing excess water to evaporate (4-5 h at room temperature), they were placed in plastic

trays on filter paper moistened with either 0.1 mM ACC or water. Each tray was put in a transparent plastic bag, but the bag was not sealed, to allow air exchange. Trays were placed in a growth chamber at 20-21 °C under fluorescent light (daylength 16 h; light intensity 10.3 $W \cdot m^{-2}$). As an additional control, intact untreated plants were kept under the same conditions.

Viruses

For inoculation of TMV (strain W U1, (26)) and ribgrass mosaic virus (RMV; synonym: Holmes' ribgrass strain of TMV), purified virus suspensions in 0.01 M phosphate buffer, pH 7.0, were used at 20 and 100 $mg \cdot l^{-1}$, respectively. The 'yellow' strain P6 (18) of cucumber mosaic virus (CMV-P6) and an isolate of CMV causing a mild green mosaic on tobacco (CMV 'green'), were maintained on *N. glutinosa*. Tobacco necrosis virus (TNV), tobacco rattle virus (TRV) and potato virus Y^N (PVY^N) were maintained on Samsun NN tobacco. Inoculum was prepared by homogenizing three symptom-bearing leaves in 15 ml phosphate buffer.

With the exception of TMV, all viruses were kindly provided by Dr J. Dijkstra, Department of Virology of the Agricultural University.

Extraction of chromatin-associated proteins

After symptoms had become evident (9-16 days, depending on the particular virus), the young expanding leaves, 4-8 cm in length, were harvested. From about 5 g deribbed leaf material, nuclei were isolated and chromatin prepared as described in detail before (27). Chromatin from senescing leaves of 14-weeks-old plants was obtained starting from 30 g of leaf material. The tissue was disrupted in 20 vol (v/w) of homogenizing buffer to dilute out substances affecting nuclear integrity in fully grown leaves.

Chromatin-associated proteins were dissociated from the DNA by resuspending the purified chromatin in 10 ml of 10 mM Tris-HCl, 3 M NaCl, 6 M urea, 1 mM $NaHSO_3$, 1% 2-mercaptoethanol, pH 8.0 (22). DNA was removed by centrifugation at 100,000 *g* for 18 h. The protein containing supernatant was used for electrophoretic analysis.

Alternatively, chromatin was extracted with 1 mM phosphate buffer pH 6.9, containing 10 M urea, 2% Nonidet P40 (NP40), 5% 2-mercaptoethanol, 1.6% Ampholine 5-7 and 0.4% Ampholine 3.5-10 at 20 °C before subjection to two-dimensional (2-D) polyacrylamide gel electrophoresis (24). Undissolved material was removed by centrifugation for 30 min at 85,000 *g*.

Analysis of chromatin-associated proteins

Chromatin-associated proteins were analyzed by electrophoresis in 10% homogeneous, or 7.5-20% and 10-17.5% linear-gradient polyacrylamide gels, containing 0.1% SDS (10). In addition, 2-D gel electrophoresis, in which proteins are separated

in the first dimension on the basis of their isoelectric points and subsequently in the second dimension on the basis of size, was performed as described by O'Farrell and O'Farrell (17).

Gels containing aliquots of about 100 μg protein per lane were stained with 0.25% Coomassie Brilliant Blue in methanol: acetic acid: water (5: 1: 4; v/v) and destained in methanol: acetic acid: water (5: 7: 88; v/v). Alternatively, gels containing about 5 μg protein per lane were developed with silver according to Morrissey (12).

Isolation and analysis of soluble proteins

Soluble proteins were isolated from leaves of 4-8 cm in length by a modification (28) of the method of Van Loon and Van Kammen (26) and analyzed by polyacrylamide gel electrophoresis as described for chromatin-associated proteins.

Preparation of TMV coat protein

TMV coat protein was prepared from a suspension of purified TMV W U1 (20 $g \cdot l^{-1}$) according to Fraenkel-Conrat (2). After dialysis and centrifugation the coat protein pellet was resuspended in 10 mM Tris-HCl, pH 8.0. Undissolved virus was removed by centrifugation for 1 h at 100,000 *g*. Samples from the clear supernatant at a concentration of 10 g coat protein$\cdot l^{-1}$ were used for electrophoretic analysis.

Identification of TMV coat protein on gels

To identify the position of TMV coat protein on 2-D gels a modified Western Blotting (15) procedure was used, in which coat protein was detected with antibodies against TMV and radioiodinated protein A.

Polypeptides present on the second dimension slab-gel were transferred by electrophoresis for a brief period (1 h, 230V) to a sheet of nitrocellulose paper (transfer buffer: 25 mM Tris, 192 mM glycine, 20% (v/v) methanol, pH 8.3). In this short time not all protein is transferred and protein remaining on the gel can be stained with silver afterwards.

After protein transfer, the nitrocellulose sheet was air-dried and subsequently incubated for 30 min at 20 °C in 30 ml of antibody buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, pH 7.4, containing 0.05% (v/v) NP40 and 0.25% (w/v) gelatin). After this time the buffer was replaced by fresh antibody buffer containing anti-TMV gammaglobulins (2 $mg.l^{-1}$), and incubation was continued for 16 h at 20 °C. Unbound antibodies were removed by washing three times for 1 h with 30 ml antibody buffer. Subsequently, the sheet was incubated in 30 ml antibody buffer containing ^{125}I-labeled protein A (55,000 $cpm \cdot ml^{-1}$) for 4 h at 20 °C. Unbound protein A was removed by three washes with 50 mM Tris-HCl, 1M NaCl, 5 mM EDTA, pH 7.4, in the presence of 0.25% gelatin (w/v) and 0.4% (w/v) sodium N-lauroylsarcosinate (Sarkosyl). After drying of the nitrocellulose sheet (1 h, 70 °C),

the position of TMV coat protein was located by autoradiography.

Anti-TMV gammaglobulins (1 $g.l^{-1}$), purified from anti-TMV rabbit serum by ammonium sulphate precipitation, were kindly provided by Dr J.Dijkstra.

Identification of CMV coat protein on gels

Strain P6 and the green isolate of CMV were purified from Samsun NN tobacco plants, using the method of Mossop *et al* (13), but without extensive purification through sucrose gradients.

The pellet obtained after high speed centrifugation was resuspended in 10 mM Tris-HCl, 1 mM EDTA, 2.5% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, pH 8.0, and heated for 5 min in a boiling water bath. Undissolved material was removed by centrifugation for 10 min at 5,000 *g*. The supernatant containing the dissociated coat protein, was used as a marker in gel electrophoresis.

Results

Plant-virus combinations examined and symptoms present on the inoculated leaves and developing top leaves 10 days after infection are summarized in Table 1. Only the young developing leaves were analyzed, because mosaic symptoms become ap-

Table 1. Characteristics of tobacco cultivar-virus combinations tested for changes in chromatin-associated proteins.

Cultivar	Virus	Temp (O°C)	Virus[a] spread	Symptoms 10 days after inoculation in the	
				Inoculated leaves	Top leaves Ranging in length from 4-8 cm
Samsun	TMV	20 - 22	+	no symptoms	mosaic, leaf malformation
White Burley	TMV	20 - 22	+	no symptoms	mosaic, leaf malformation
Samsun	TMV	31 - 32	+	no symptoms	mosaic, leaf malformation
Samsun NN	TMV	31 - 32	+	no symptoms	mosaic
Samsun NN	TMV	20 - 22	-	necrotic lesions	no symptoms
Samsun	CMV-P6	20 - 22	+	yellow chlorotic spots	bright yellow mosaic
Samsun NN	CMP-P6	20 - 22	+	yellow chlorotic spots	bright yellow mosaic
Samsun	CMV-green	20 - 22	+	no symptoms	mild green mosaic
Samsun NN	CMV-green	20 - 22	+	no symptoms	mild green mosaic
Samsun	RMV	20 - 22	+	thin necrotic lines	light mottling
Samsun NN	RMV	20 - 22	-	and necrotic ring lesions	no symptoms
Samsun	RMV	31 - 32	+	no symptoms[b]	no symptoms[b]
Samsun NN	RMV	31 - 32	+	no symptoms[b]	no symptoms[b]
Samsun	TRV	20 - 22	+	necrotic lesions	light crinkling
Samsun NN	TRV	20 - 22	+	necrotic lesions	light crinkling
Samsun	TNV	20 - 22	-	necrotic lesions	no symptoms
Samsun NN	TNV	20 - 22	-	necrotic lesions	no symptoms
Samsun	PVY^N	20 - 22	+	vein clearing and necrosis	vein clearing
Samsun NN	PVY^N	20 - 22	+	vein clearing and necrosis	vein clearing

a) + : systemic spread ; - = localization in inoculated leaves

b) presence of virus established by infectivity assay on *N. glutinosa*

parent only on these leaves. Absence of symptoms in top leaves of plants infected with viruses causing either symptomless systemic infection (RMV, 31-32 °C) or a localized hypersensitive reaction (Samsun NN-TMV, 20-22 °C; -TNV, 20-22 °C) provided conditions to test for changes independent of symptom development but related to viral multiplication and the development of systemic acquired resistance, respectively.

Changes in chromatin-associated proteins after infection with TMV

Comparison of the electrophoretic patterns of the chromatin-associated proteins from TMV-infected tobacco's revealed that in mosaic-diseased White Burley grown at 20-22 °C the same change occurred as found earlier in Samsun, namely a new protein with an approximate molecular mass of 116 kDa (Fig 1a). The same new protein was also evident in TMV-infected Samsun at 31-32 °C. However, it was not

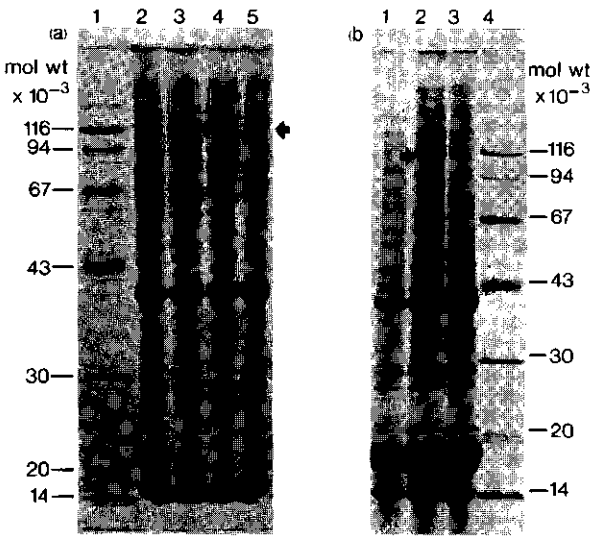


Fig. 1: Electrophoretic patterns of chromatin-associated proteins in SDS-containing polyacrylamide gels stained with Coomassie Blue. (a) 10% homogeneous polyacrylamide gel with proteins from buffer-treated (lanes 2 and 4) and TMV-infected Samsun and White Burley tobacco (lanes 3 and 5, respectively). Lane 1 contains marker proteins: β-galactosidase (116,000), phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100) and α-lactalbumin (14,400); (b) 10-17.5% linear gradient polyacrylamide gel containing chromatin-associated proteins from TMV-infected Samsun NN grown at 20-22 °C (lane 1) and TMV-infected Samsun and Samsun NN tobacco grown at 31-32 °C (lanes 2 and 3, respectively). Lane 4 contains the marker proteins. Arrows indicate the new 116 kDa band.

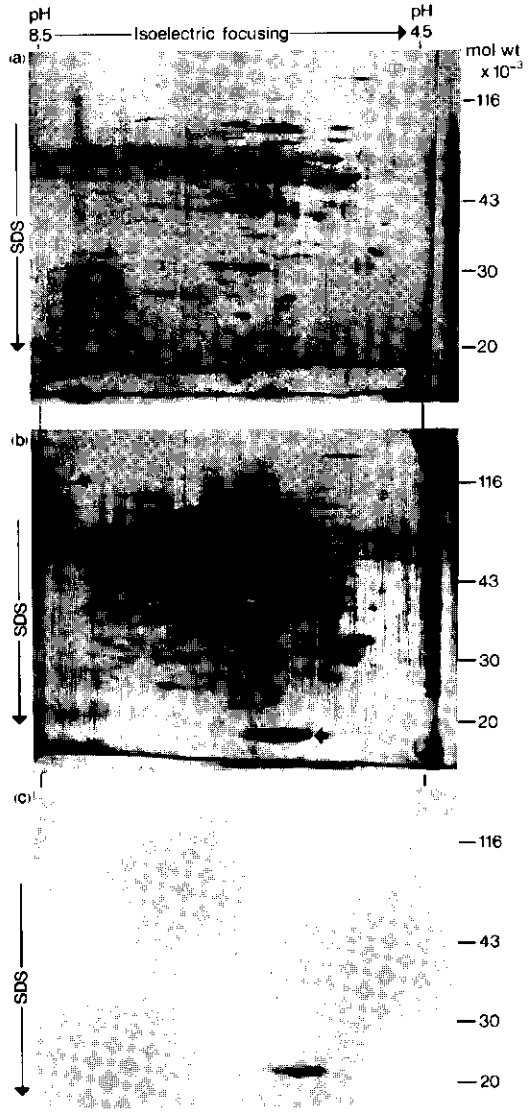


Fig. 2: Two dimensional polyacrylamide gel electrophoretic patterns of chromatin-associated proteins from (a) buffer-treated and (b) TMV-infected Samsun tobacco. The pH gradient of the isoelectric focusing gel was from pH 4.5 (right) to pH 8.5 (left). A total of approximately 5 μg protein was loaded on each isoelectric focusing gel. Lanes at the right contain samples of total chromatin-associated proteins run in the second dimension only. Gels were stained with silver. Differences in staining intensities between (a) and (b) are caused by the minor variations in the amounts of protein applied and developing time. (c) Autoradiograph obtained after Western Blotting of the same gel shown in (b). Arrows indicate the new 116 kDa and 20 kDa spots.

discernable in the profile from mosaic-diseased Samsun NN at 31-32 °C, not even in gradient gels, which give greater resolution than homogeneous polyacrylamide gels particularly for high-molecular-weight proteins (Fig 1b). Neither were any new proteins found in the non-infected upper leaves of TMV-inoculated Samsun NN plants which had been grown at 20-22 °C and whose lower leaves exhibited a hypersensitive reaction (Fig 1b). No differences were noted between protein patterns of leaves from non-infected Samsun, Samsun NN or White Burley plants grown at 20-22 °C or 31-32 °C.

When chromatin protein extracts from TMV-infected Samsun (Fig 2b) or White Burley leaves (gel not shown) were subjected to 2-D gel electrophoresis, the 116 kDa protein was found at the very basic end of the gel, at around pH 8.5. This high isoelectric point indicates that the 116 kDa protein must be relatively basic. Furthermore, these 2-D gels as well as those of TMV-infected Samsun NN plants grown at 31-32 °C (gel not shown) revealed an additional new spot with an approximate molecular mass of 20 kDa and a pI ranging from 4.9 to 5.9. As shown in Fig 2c, this broad spot reacted with anti-TMV gammaglobulins, indicating that it contained TMV coat protein. Both in the silver-stained pattern (Fig 2b) and in the antiserum-specific blotting pattern (Fig 2c), the TMV coat protein spot appeared to consist of a number of discrete subspots, each with a slightly different isoelectric point. Control experiments in which purified TMV coat protein was similarly analysed by 2-D gel electrophoresis, showed only one discrete spot at pI 4.9. 2-D patterns of TMV-infected Samsun NN plants grown at 20-22 °C showed no differences with those from non-infected Samsun NN plants (gels not shown).

Since during TMV multiplication viral coat protein is produced in large excess and accumulates in the cytoplasm (25), we investigated whether free coat protein could associate non-specifically with the chromatin during nuclei isolation. To this end, a crude nuclear pellet prepared from 5 g of healthy leaves was resuspended in buffer containing purified TMV coat protein (final concentration 2.5 $g.l^{-1}$) and incubated for 1 h before continuing chromatin protein isolation. Under these conditions, no spot of TMV coat protein was discernable among the chromatin-associated proteins upon separation on 2-D gels.

Changes in chromatin-associated proteins after infection with other viruses

In Samsun NN tobacco, the occurrence of a mild green mosaic due to CMV 'green' was associated with a doubling of a band with an approximate molecular mass of 28 kDa (Fig 3a). This doubling indicated that, besides a 28 kDa protein present in non-infected plants, a new protein of about the same molecular mass, but migrating slightly further into the gel, became present as a result of the infection. An intensification of the corresponding band in the pattern of CMV 'green' infected Samsun leaves was suggestive of the presence of some of the new protein in this cultivar too, but two separate bands were never observed. The new 28 kDa band was not found in

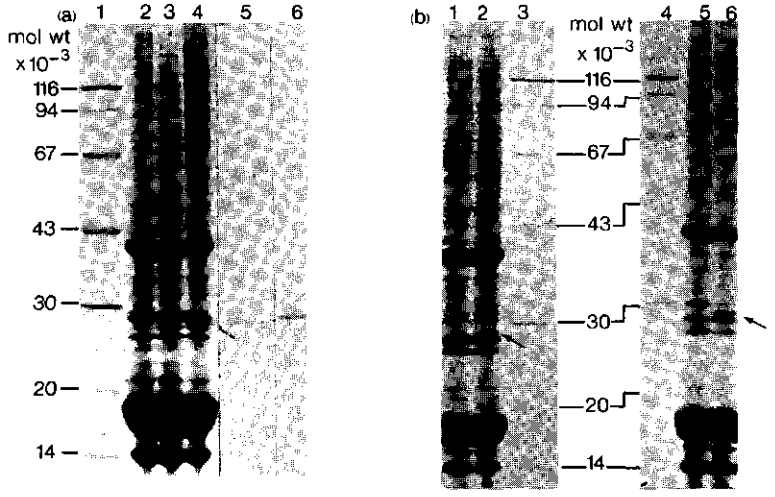


Fig. 3: Electrophoretic patterns of chromatin-associated proteins in SDS-containing polyacrylamide gels stained with Coomassie Blue. (a) 10-17.5% linear gradient gels with proteins from buffer-treated Samsun (lane 2), and CMV 'green' infected Samsun and Samsun NN tobacco (lanes 3 and 4, respectively). Lanes 5 and 6 contained the coat protein of CMV 'green' and CMV P6, respectively. Lane 1 Contains the marker proteins. Arrow indicates the new 28 kDa band; (b) 7.5-20% linear gradient gels with proteins from buffer-treated and CMV P6-infected Samsun (lanes 1 and 2), or Samsun NN tobacco (lanes 5 and 6). Lanes 3 and 4 contain marker proteins. Arrows indicate the new 29 kDa band.

leaves showing a bright yellow mosaic due to infection with CMV strain P6. However, when using a steeper gradient (7.5-20% instead of 10-17.5% acrylamide), both in Samsun and Samsun NN a new band with an approximate molecular mass of 29 kDa was revealed; this new protein migrated slightly more slowly than the 28 kDa band present in non-infected plants (Fig 3b). The positions of the newly induced 28 and 29 kDa proteins coincided with those of the coat proteins of CMV 'green' and CMV P6, respectively (Fig 3a). These observations clearly suggest that, like in the case of TMV, viral coat protein is present among the chromatin-associated proteins of CMV-infected tobacco.

Two-dimensional gel electrophoresis did not reveal any differences between the profiles of healthy and CMV-infected plants (gels not shown), possibly because the pI of CMV coat protein falls outside the pH range covered by the isoelectric focusing gel.

RMV, a member of the tobamo virus group, induced slight necrosis on Samsun NN and a semi-systemic yellow ringspotting on Samsun tobacco at 20-22 °C, but caused symptomless systemic infection in both cultivars at 31-32 °C. No changes in the chromatin-associated protein profile became evident under any of these conditions (Fig 4a). Although local lesion assay on *N. glutinosa* verified the presence of

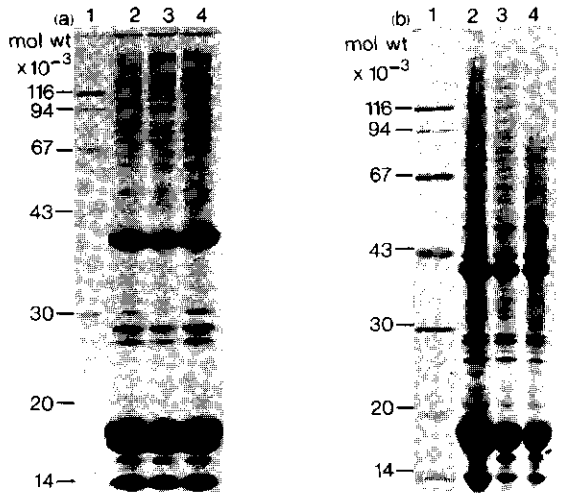


Fig. 4: Electrophoretic patterns of chromatin-associated proteins in 10-17.5% linear gradient polyacylamide gels containing SDS, stained with Coomassie Blue: (a) protein from buffer-treated (lane 2) and RMV-infected Samsun NN tobacco grown at 20-22 °C (lane 3) or 31-32 °C (lane 4). Lane 1 Contains marker proteins; (b) protein from buffer-treated (lane 2), PVY^N- (lane 3) and TRV-infected (lane 4) Samsun tobacco grown at 20-22 °C. Lane 1 Contains marker proteins.

substantial amounts of infectious RMV in both the inoculated and the systemically infected leaves at 31-32 °C, no viral coat protein could be detected in 2-D gels among the chromatin-associated proteins. Neither was any free coat protein detectable in the soluble leaf protein fraction by electrophoresis in SDS-containing gels.

Infection with TNV did not induce any changes in the chromatin-associated protein profile of the symptomless young, upper leaves. Neither did PVY^N or TRV lead to any clear qualitative changes. However, there was a quantitative loss in high-molecular-weight proteins, the more so in the TRV-infected leaves which showed incipient necrosis (Fig 4b).

Changes in chromatin-associated proteins during leaf ageing or senescence

Treatment of young, developing leaves with ACC did not induce any of the changes observed after infection with TMV or CMV (Fig 5a). Although ethylene production was stimulated more than 20-fold within 48 h, there was only a slight loss of chlorophyll (−12%) in ACC-infiltrated leaves compared to that of the water-infiltrated controls. At this stage of development, the leaves were probably refractory to the senescence-inducing action of ethylene. However, both the water and the ACC treatments induced a marked increase in intensity of a band with an approximate molecular mass of 31 kDa (Fig 5a). Such a change was also evident in non-in-

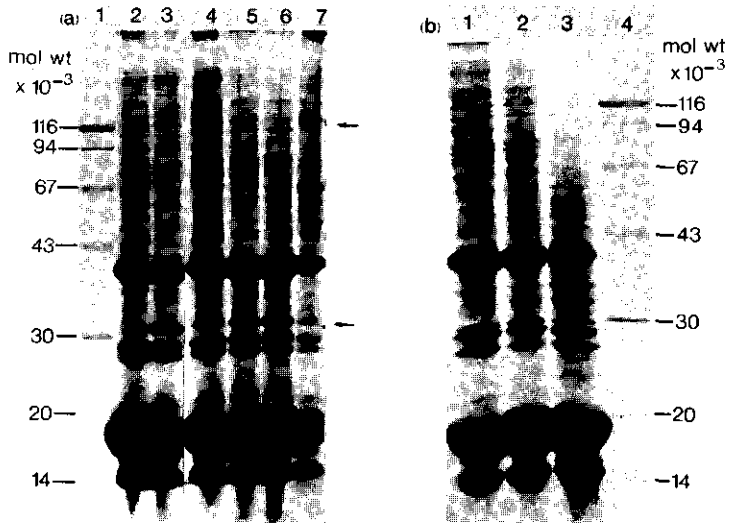


Fig. 5: Electrophoretic patterns of chromatin-associated proteins in 10-17.5% linear gradient polyacrylamide gels containing SDS, stained with Coomassie Blue: (a) proteins from non-infected Samsun NN tobacco grown at 20-22 °C under natural light (lane 2) or at 31-32 °C under fluorescent light only (lane 3), and from non-infected Samsun tobacco grown at 20-22 °C under fluorescent lights only (lane 4); half leaves vacuum-infiltrated with water (lane 5) or 0.1 mM ACC (lane 6) and incubated under the same conditions. Lane 1 Contains marker proteins and lane 7 chromatin-associated proteins from TMV-infected Samsun plants grown at 20-22 °C under natural light. Arrows indicate the positions of the 31 kDa band in lanes 3, 4, 5 and 6, and of the new 116 kDa band in lane 7. (b) proteins from young, 4-8 cm long leaves from non-infected Samsun tobacco of 7 weeks (lane 1) or 14 weeks old (lane 2) and from the yellowing, senescing leaves from 14 weeks-old plants (lane 3). Lane 4 contains marker proteins.

fected Samsun NN plants when, similar to the conditions of incubation, these were grown under fluorescent lights only, both at 20-22 and 31-32 °C (Fig 5a). These observations indicate that, whereas increased ethylene levels in the leaf had no effect, a change in light conditions influenced chromatin-associated protein profiles in a defined way.

In order to determine if the changes in chromatin-associated proteins which occurred after virus infection also occurred during normal leaf senescence, the protein profiles of old, yellowing leaves from 14 weeks old Samsun plants which were about to flower were examined. As shown in Fig 5b, the chromatin-associated protein profile of the upper, 4-8 cm long leaves was similar to that of the young leaves from 7-weeks-old plants. In contrast, the lower, yellow leaves showed several differences, notably an almost complete absence of high molecular-weight proteins. This effect shows a striking resemblance to that of TRV on the protein profile in systemically-

infected leaves (Fig 4b), suggesting that at least TRV infection induces changes similar to senescence. However, the senescing leaves from healthy plants did not exhibit any of the changes characteristic of TMV or CMV infection.

Discussion

Results presented here show that in tobacco when virus infection leads to the development of systemic mosaic symptoms, specific changes occur in the profile of the chromatin-associated proteins. Such changes do not occur when infection results in systemic necrosis (TRV, PVY^N, and RMV on Samsun) or when the virus remains localized in the inoculated leaves and the upper part of the plant developsacquired systemic resistance (TNV, and TMV or RMV on Samsun NN at temperatures below 28 °C).

Treatment of young leaves with ACC did not induce premature ageing, nor any of the changes induced by infection with TMV or CMV and neither did natural senescence. Thus, the changes observed in mosaic diseased leaves are not related to the senescence of the light-green leaf areas. Instead, the observed changes appeared to be virus-dependent with both TMV and CMV inducing specific changes, apparently related to the expression of systemic mosaic symptoms.

In both Samsun and White Burley, infection with TMV induced a new protein of about 116 kDa. 2-D gel electrophoresis revealed an additional change in these two cultivars, which was also apparent in TMV-infected Samsun and Samsun NN at 32 °C. This change consisted of the accumulation of a major protein with a molecular mass of about 20 kDa, which was identified by Western Blotting as TMV coat protein. The reason that the TMV coat protein was not revealed by one-dimensional SDS-polyacrylamide gel electrophoresis may be due to the different methods used to dissociate the proteins from the chromatin for each type of gel electrophoresis (cf. 27). Although TMV coat protein has a molecular mass of 17.5 kDa (11), it is commonly found to migrate as a 20 kDa protein in gradient gels such as used in this investigation.

A relationship between TMV coat protein properties and symptom expression in tobacco has been suggested previously. Von Sengbusch (21) suggested that the induction of yellow mosaic symptoms by certain mutants of the virus was a function of charge alterations of the coat protein. This hypothesis was supported by the findings of Jockusch (8, 9) that all mutants inducing yellow symptoms possessed temperature sensitive coat proteins with unusual solubility properties. Recently, Fraser (3) reported that the effectiveness with which the *N'* gene restricts the rate of spread of different TMV strains and mutants appears to be affected by the nature of the virus coat protein.

Our findings that TMV coat protein is associated with the host chromatin may provide a molecular basis for these observations. In this respect, the presence of several isoelectric forms of the coat protein may be particularly significant. Additional

support may be derived from the finding that after systemic infection at 31-32 °C with another tobamo virus, RMV, symptoms were absent and neither the 116 kDa protein, nor RMV coat protein could be found associated with chromatin.

After infection of Samsun NN or Samsun tobacco with CMV P6 only one new protein band of about 29 kDa could be detected, whereas infection with CMV 'green' resulted in one new band of about 28 kDa. Coat proteins of the respective virus strains co-migrated with these bands, indicating that also after CMV infection coat protein may be involved in symptom development.

Such involvement has also been suggested earlier. By constructing pseudo-recombinants it has been shown that some host reactions are determined by CMV RNA 3 (19, 29), which besides the gene for aphid transmissibility contains the coat protein gene (14). It has also been reported that upon infection with different CMV strains symptom severity is correlated with the rate of synthesis of the coat protein (5). The severe yellow mosaic-inducing strain P6 synthesizes considerably more coat protein than a mild strain (20). Since symptoms of CMV P6 are usually more severe in Samsun NN than in Samsun plants (25), these conditions may explain why in CMV 'green' infected Samsun plants the presence of coat protein could not be established with certainty.

The presence of coat proteins of both TMV and CMV in host chromatin from mosaic-diseased leaves, suggests that viruses may employ this as a strategy to affect host plant metabolism. Significantly, in tobacco infected with alfalfa mosaic virus, viral coat protein has been detected in the nucleus recently by immuno-electron microscopy (Van Vloten-Doting, personal communication). The way by which viral coat proteins might influence symptom development may thus be sought in their ability to bind to host DNA. Since, at least under our *in vitro* conditions, purified TMV coat protein did not associate with the chromatin in nuclei from healthy tobacco leaves, non-specific adsorption of viral coat protein to host chromatin can be ruled out. This suggests that coat protein, by binding to specific sites on the chromatin, could, at least in principle, interfere specifically with the normal pattern of plant growth and development.

It seems unlikely, however, that coat protein is the only or primary determinant for symptom induction in virus-infected tobacco. For instance, the 'masked' strain of TMV (7), which does not induce visible symptoms, has a coat protein identical to that of the common strain (30). Other examples may be found among the the chemically-induced TMV mutants that have no amino acid substitutions in the coat protein but nevertheless induce very different symptoms (6). A probable minor role for coat protein has also been demonstrated for CMV. Infection with a pseudo-recombinant obtained by mixing the two large RNAs 1 and 2 of tomato aspermy virus with the small, coat-protein-encoding RNA 3 of CMV resulted in virions encapsidated in CMV coat protein, but symptoms were those of tomato aspermy virus (4). More gen-

erally, genetic information for symptom expression and coat protein is not linked in viruses with a divided genome.

Thus, proteins other than viral coat proteins are obviously important in symptom expression. In this respect the new protein of 116 kDa in TMV-infected plants may also play a role. As it was found only in severely mosaic-diseased plants infected with TMV, like the coat protein it shows virus-specificity. This relationship will be further analyzed in a subsequent paper.

References

1. Aharoni, N. and Lieberman, M. (1979). Ethylene as a regulator of senescence in tobacco leaf discs. *Plant Physiology* 64, 801-804.
2. Fraenkel-Conrat, H. (1957). Degradation of tobacco mosaic virus with acetic acid. *Virology* 4, 1-4.
3. Fraser, R. S. S. (1983). Varying effectiveness of the *N'* gene for resistance to tobacco mosaic virus in tobacco infected with virus strains differing in coat protein properties. *Physiological Plant Pathology* 22, 109-119.
4. Habili, N. and Francki, R. I. B. (1974). Comparitive studies on tomato aspermy and cucumber mosaic viruses. III. Further studies on relationship and construction of a virus from parts of two viral genomes. *Virology* 61, 443-449.
5. Havránek, P. (1978). Use of quantitative immunoelectrophoresis in cucumber mosaic virus assay. II. Strain- and isolate-specific modifications of the antigenic complex. *Phytopathologische Zeitschrift* 93, 5-11.
6. Hennig, B. and Wittmann, H. G. (1972). Tobacco mosaic virus: Mutants and strains. In: *Principles and techniques in plant virology*. Ed. by C. I. Kado and H. O. Agrawal, pp 546-594. Van Nostrand-Reinhold, New York.
7. Holmes, F. O. (1934). A masked strain of tobacco mosaic virus. *Phytopathology* 24, 845-873.
8. Jockusch, H. (1966). Temperatursensitive Mutanten des Tabaksmosaikvirus. II. *In vitro*-Verhalten. *Zeitschrift für Vererbungslehre* 98, 344-362.
9. Jockusch, H. and Jockusch, B. (1968). Early cell death caused by mutants with defective coat proteins. *Molecular and general Genetics* 102, 204-209.
10. Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685.
11. Matthews, R. E. F. (1981). In: *Plant Virology*, 2nd Edition. Academic Press, New York and London.
12. Morrissey, J. H. (1981). Silver stain for proteins in polyacrylamide gels. A modified procedure with enhanced uniform sensitivity. *Analytical Biochemistry* 117, 307-310.
13. Mossop, D. W., Francki, R. I. B. and Grivell, C. J. (1976). Comparative studies on Tomato Aspermy and Cucumber Mosaic Viruses. V: Purification and properties of a cucumber mosaic virus inducing severe chlorosis. *Virology* 74, 544-546.
14. Mossop, D. W. and Francki, R. I. B. (1977). Association of RNA-3 with aphid transmission of cucumber mosaic virus. *Virology* 81, 171-181.
15. Neal Burnette, W. (1981). Western Blotting: Electrophoretic transfer of proteins from sodium dodecyl sulphate-polyacrylamide gels to unmodified nitrocellulose and radiographic

detection with antibody and radioiodinated protein A. *Analytical Biochemistry* 112, 195-203.
16. Nilsson-Tillgren, T., Kolehmainen-Sevéus, L. and Wettstein, D. von (1969). Studies on the biosynthesis of TMV. I. A system approaching a synchronized virus synthesis in a tobacco leaf. *Molecular and General Genetics* 104, 124-141.
17. O'Farrell, P. H. and O'Farrell, P. Z. (1977). Two dimensional polyacrylamide gel electrophoretic fractionation. In: *Methods in Cell Biology* XVI, Ed. by G. Stein, J. Stein and C. J. Kleinsmith, pp 407-420. Academic Press, New York.
18. Price, W. C. (1934). Isolation and study of some yellow strains of cucumber mosaic. *Phytopathology* XXIV, 743-761.
19. Rao, A. L. W. and Francki, R. I. B. (1982). Distribution of determinants for symptom production and host range on the three RNA components of cucumber mosaic virus. *Journal of general Virology* 61, 197-205.
20. Roberts, P. L. and Wood, K. R. (1981). Comparison of protein synthesis in tobacco systemically infected with a severe or a mild strain of cucumber mosaic virus. *Phytopathologische Zeitschrift* 102, 257-265.
21. Sengbusch, P. von (1965). Aminosäureaustausche und Tertiärstruktur eines Proteins. Vergleich von Mutanten des Tabaksmosaikvirus mit serologischen und physikochemischen Methoden. *Zeitschrift für Vererbungslehre* 96, 364-386.
22. Shaw, L. M. J. and Huang, R. C. C. (1970). A description of two procedures which avoid the use of extreme pH conditions for the resolution of components isolated from chromatins prepared from pig cerebellar and pituitary nuclei. *Biochemistry* 9, 4530-4542.
23. Thompson, W. F. and Murray, M. G. (1981). The nuclear genome: structure and function. In: *The Biochemistry of Plants, A Comprehensive Treatise*. Vol.6: Proteins and Nucleic Acids, Ed. by A. Marcus, pp 52-81. Academic Press, New York and London.
24. Trewavas, A. (1979). Nuclear phosphoproteins in germinating cereal embryos and their relationship to the control of mRNA synthesis. In: *Recent advances in the biochemistry of cereals*, Ed. by D. L. Laidman and R. C. Wyn Jones, pp 175-208. Phytochemical Society Symposium 16.
25. Van Loon, L. C. (1975). Polyacrylamide disc electrophoresis of the soluble leaf proteins from *Nicotiana tabacum* var 'Samsun' and 'Samsun NN', IV: Similarity of qualitative changes of specific proteins after infection with different viruses and their relationship to acquired resistance. *Virology* 67, 566-575.
26. Van Loon, L. C. and Van Kammen, A. (1970). Polyacrylamide disc electrophoresis of the soluble leaf proteins from *Nicotiana tabacum* var 'Samsun' and 'Samsun NN'. II: Changes in protein constitution after infection with tobacco mosaic virus. *Virology* 40, 199-211.
27. Van Telgen, H. J. and Van Loon, L. C. (1983). Isolation and electrophoretic analysis of chromatin-associated proteins from virus-infected tobacco leaves. *Zeitschrift für Pflanzenphysiologie* 112, 171-180.
28. Van Telgen, H. J., Van der Zaal, E. J. and Van Loon, L. C. (1984). Some characteristics of the association of the 116 kDa protein with host chromatin in TMV-infected tobacco leaves. *Physiological Plant Pathology*, in press.

29. Van Vloten-Doting, L. and Jaspars, E. M. J. (1977). Plant covirus systems: Three component systems. In: *Comprehensive Virology* 11, Ed. by H. Fraenkel-Conrat and R. R. Wagner, 1-53. Plenum Press, New York.
30. Zaitlin, M. (1979). How viruses and viroids induce disease. In: *Plant Disease, An Advanced Treatise*, Vol. IV. How pathogens induce disease, Ed. by J. G. Horsfall and E. B. Cowlings, 257-271. Academic Press, New York.

Chapter 4
Some Characteristics of the Association of the 116 kDa Protein with Host Chromatin in Tobacco Leaves Infected with Tobacco Mosaic Virus

Summary

Properties of the new 116 kDa protein associated with chromatin in mosaic-diseased leaves of tobacco mosaic virus (TMV) infected tobacco were investigated. Whereas TMV was detected by ELISA in the systemically infected leaves by 96 h, the 116 kDa protein became discernable between 120-144 h after inoculation. This moment coincided with the appearance of the first visible symptoms (vein clearing). The 116 kDa protein was also present both in the soluble protein and in the sedimentable membrane fraction. However, since its concentration in nuclei was calculated to be about eight-fold higher than in the cytoplasm, it appears to be preferentially associated with chromatin. Moreover, its dissociation from the chromatin required salt, in contrast to TMV coat protein which was released by urea only. This indicates that the 116 kDa protein is bound more tightly to chromatin than TMV coat protein. This behaviour of the 116 kDa protein as a real chromatin protein suggests that it may play a regulatory role in pathogenesis and symptom expression in developing leaves.

Introduction

The occurrence of light and dark green mosaic symptoms on the young, developing leaves of tobacco plants infected with tobacco mosaic virus (TMV) is accompanied by the appearance of two new chromatin-associated proteins with apparent molecular masses of 20 kDa and 116 kDa, respectively (17, 18). The 20 kDa protein has been identified serologically as TMV coat protein (18). The 116 kDa protein appeared TMV-specific, as it did not occur in cucumber mosaic virus (CMV) infected tobacco, although CMV coat protein was present among the chromatin-associated proteins of mosaic-diseased leaves. Furthermore, it was not induced in healthy tobacco leaves during natural senescence or artificial ageing (18), making it unlikely that the 116 kDa protein is involved in changes related to the senescence occurring in the light green leaf areas (8).

It thus became of interest to further study the relationship between TMV infection and the occurrence of the 116 kDa protein. To this end, its rate of appearance after infection and the specificity of its association with the host chromatin were investigated.

Materials and methods

Plants and virus

Tobacco plants (*Nicotiana tabacum* L.) cv. Samsun were grown in a greenhouse as

described previously (17). When 7 to 8 weeks old, plants were inoculated on two fully expanded leaves with purified TMV W U1 (20 mg.l^{-1}) (18).

Isolation of chromatin-associated proteins

At different times after inoculation young leaves (4-8 cm long) from 20 plants were harvested and chromatin was prepared as described before (17). Chromatin-associated proteins were dissociated from the purified chromatin by resuspension in 10 mM Tris-HCl, 3 M NaCl, 6 M urea, 1 mM $NaHSO_3$, 1% (v/v) 2-mercaptoethanol pH 8.0 (14), and DNA was removed by centrifugation for 18 h at 100,000 *g*. Alternatively, acid-soluble chromatin proteins were extracted according to Towill and Noodén (15).

To investigate the effects of NaCl and urea on the release of protein from the chromatin, from 100 plants inoculated with TMV 10 days earlier, chromatin was prepared and divided into equal portions. Aliquots were extracted with buffer containing a series of NaCl or urea concentrations.

Isolation of soluble leaf proteins

From duplicate plants, young leaves similar to those used for the isolation of chromatin-associated proteins were washed with demineralized water and deribbed. A total of 3 g leaf material was ground in a mortar on ice in 10 ml 0.1 M Tris-HCl buffer pH 8.0, containing 0.5 M sucrose, 5 mM $MgCl_2$, 0.1% (w/v) cysteine and 0.1% (w/v) ascorbic acid (16). The homogenate was filtered through gauze. The mortar was rinsed with 5 ml buffer and the washing fluid similarly filtered. The combined filtrates were centrifuged for 10 min at 360 *g* and the turbid, green supernatant thus obtained was subsequently centrifuged for 3 h at 72,000 *g*. To 5 ml of the clear, yellow supernatant 3 vol of cold acetone were added and the mixture was stored at -20 °C for at least 1 h. The resulting precipitate was collected by centrifugation for 10 min at 10,000 *g*, dried under vacuum, dissolved in SDS sample buffer (10 mM Tris-HCl, 1mM EDTA, 2.5% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, pH 8.0), heated for 3 min in a boiling water bath and used for electrophoretic analysis.

Isolation of membrane-associated proteins

Membrane-associated proteins were isolated from the 72,000 *g* pellet obtained during the isolation of soluble leaf proteins. The green pellet was resuspended in 25 ml 75 mM Tris-HCl buffer pH 8.0, containing 0.35 M sucrose, 3.5 mM $MgCl_2$, 0.075%(w/v) cysteine and 0.075%(w/v) ascorbic acid and the suspension recentrifuged at 72,000 *g*. The resulting washed pellet was resuspended in 2 ml SDS sample buffer, and treated as described in the previous section.

Determination of leaf protein content

Total leaf protein content was estimated by grinding 1 g of deribbed leaf material in a mortar on ice in 10 ml acetone, containing 1% (w/v) ascorbic acid. The homogenate was poured into a centrifuge tube and the mortar was rinsed two times with 5 ml 80% acetone containing 1% ascorbic acid, which were added to the homogenate. The pellet obtained after centrifugation for 10 min at 3,000 *g* was resuspended in 15 ml 0.1 M NaOH and the mixture recentrifuged. The resulting pellet was re-extracted with 10 ml 0.1 M NaOH, the mixture again centrifuged, and the supernatant combined with the previous one. To the combined supernatants an equal volume of ice-cold 10% trichloroacetic acid (TCA) was added. Precipitated protein was collected by centrifugation for 10 min at 3,000 *g* and subsequently dissolved in 10 ml 0.1 M NaOH. Protein was then determined using the method of Bradford (1).

Electrophoretic analysis of proteins

Heated protein samples in SDS sample buffer were analyzed in 7.5-17.5% linear gradient polyacrylamide gels containing 0.1% SDS (7). Gels were stained with 0.25 % Coomassie Brilliant Blue in methanol: acetic acid: water (5: 1: 4,v/v) and destained in methanol: acetic acid: water (5: 7: 88,v/v). Alternatively, the silver staining method of Morrissey (10) was used.

Peptide analysis

To characterize specific protein bands, slices were cut from duplicate gels and incubated with 0.02-0.10 μg protease V8 from *Staphylococcus aureus* (Miles 36.900) following the procedure described by Cleveland *et al* (4). The peptides resulting from the limited proteolysis were analyzed by electrophoresis in 7.5-20% linear gradient polyacrylamide gels containing 0.1% SDS. Gels were stained with silver as mentioned above.

Determination of TMV content

The course of TMV infection of the young, developing leaves was monitored by measuring the amount of TMV by the sensitive ELISA technique (3). From the 4-8 cm long leaves, discs (1 cm in diameter) were punched. Three discs were ground in an all-glass Potter homogenizer in 3 ml phosphate-buffered saline (PBS), containing 0.05% (v/v) Tween 20 and 2% (w/v) polyvinylpyrrolidone (PVP). The homogenate was diluted 10 times with the same buffer and the amount of TMV determined using a purified virus preparation as a standard. Anti-TMV gammaglobulins (1 $g.l^{-1}$) were kindly provided by Dr J. Dijkstra, Department of Virology of the Agricultural University.

Preparation of TMV coat protein

TMV coat protein was prepared as described elsewhere (18). The purified protein

was dissolved in SDS sample buffer and, after heating, used for electrophoretic analysis.

Estimation of nuclear and cytoplasmic volumes

Small pieces from leaves varying in length from 4 to 8 cm were fixed in 5% glutaraldehyde, buffered in 0.1 M Na-cacodylate, pH 7.0. After staining with 1% $KMnO_4$, the samples were dehydrated and embedded in Epon using conventional techniques. Semi-thin sections were examined by light microscopy and nuclear and cytoplasmic volumes of the mesophyll cells were determined with a Kontron MOP-30 image analyzer.

Results

Time course of the appearance of TMV and the 116 kDa protein in chromatin from developing leaves

The time course of TMV infection and multiplication in systemically infected, young, developing leaves of 4-8 cm in length, as measured by ELISA, is shown in Fig 1. After the inoculation of two fully expanded leaves, the first TMV could be de-

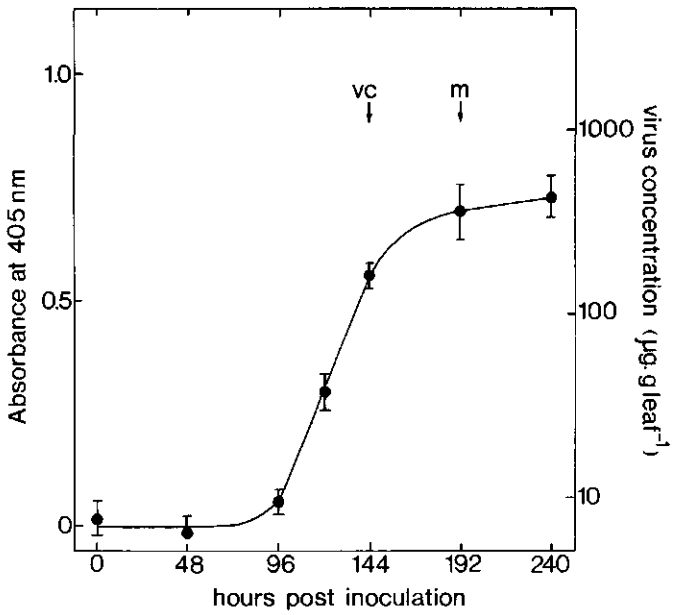


Fig. 1: Time course of TMV multiplication, as measured by ELISA, in 4-8 cm long upper leaves from Samsun tobacco plants inoculated on two fully expanded leaves at t= 0 h. Arrows indicate the times of first vein clearing (vc) and mosaic symptoms (m) on the developing leaves.

tected in the top leaves as early as 96 h later, followed by a sharp increase in virus content during the next 48 h. The first visible symptoms (vein clearing) appeared 144 h after inoculation, and the first mosaic symptoms became visible about 48 h later. Due to the presence of minor bands around the position of the 116 kDa protein in the chromatin-associated protein profiles of non-infected plants, the exact time of appearance of the 116 kDa protein after infection was difficult to establish. However, in different experiments, by 144 h after inoculation the 116 kDa protein was clearly recognizable as a doubling of a band already present (Fig 2a). At this time, vein clearing had become evident in the leaves and also TMV coat protein was first detectable in the gel profiles of the soluble protein fraction (Fig 2b). Since with ELISA the presence of viral coat protein was demonstrated from 96 h after inoculation onwards, gel electrophoresis appears to be considerably less sensitive to recognize small quantities of this protein. In the chromatin protein fraction, the 116 kDa protein was present as a major band at 192 h (mosaic symptoms) and remained so at least up to 240 h. The

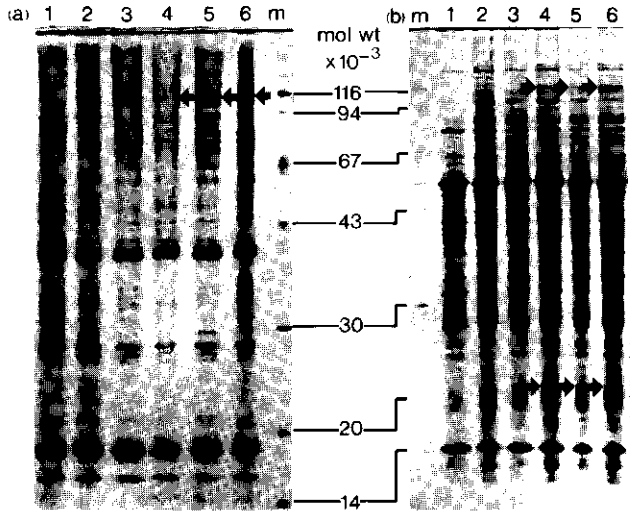


Fig. 2: Electrophoretic patterns of (a) chromatin-associated and (b) soluble proteins in 0.1% SDS-containing 7.5-17.5% linear gradient polyacrylamide gels stained with silver. Proteins were isolated from 4-8 cm long leaves of Samsun tobacco plants at 0 h (lane 1), 72 h (lane 2), 120 h (lane 3), 144 h (lane 4), 192 h (lane 5) and 240 h (lane 6) after inoculation of two fully expanded leaves with TMV. Each lane contains about 5 μg of protein. Arrows indicate additional protein bands. The lanes marked M contain marker proteins: β-galactosidase (116,000), phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100) and α-lactalbumin (14,400).

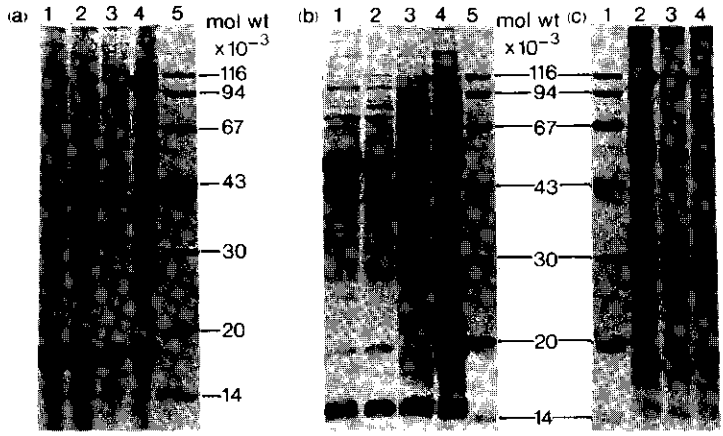


Fig. 3: Electrophoretic patterns of (a) chromatin-associated, (b) soluble and (c) membrane-associated proteins from healthy and TMV-infected Samsun tobacco plants in 0.1% SDS-containing 7.5-17.5% linear gradient polyacrylamide gels stained with Coomassie Blue. In (a) and (b) proteins were isolated from fully-expanded, inoculated leaves 120 h after inoculation (lane 2) and from mosaic-diseased leaves 264 h after inoculation (lane 4). The respective control proteins from non-infected plants are shown in lanes 1 and 3, respectively. Lane 5 contains marker proteins. In (c) membrane-associated proteins were isolated from the inoculated leaves 120 h after inoculation (lane 3) and from the mosaic-diseased leaves 264 h after inoculation (lane 4). Lane 2 contains the proteins from fully-expanded leaves from non-infected plants and lane 1 the marker proteins. Each lane contained about 100 μg of protein. Arrows indicate the positions of the 116 kDa and TMV coat protein.

appearance of the 116 kDa protein was not confined to the systemically infected leaves: 120 h after inoculation it was also present in the chromatin of the inoculated leaves which, in spite of a high virus content, remained symptomless (Fig 3a).

Subcellular localization of the 116 kDa protein

At the time the 116 kDa protein had become apparent in the chromatin-associated protein profile (Figs 2a, 3a), a new band of the same molecular mass was discernable in the profile of the soluble protein fraction (Figs 2b, 3b). As in the chromatin fraction, this soluble 116 kDa protein increased in amount with time. Limited digestion of the 116 kDa protein in slices from both chromatin-associated- and soluble protein-containing gels with protease V8 resulted in essentially identical peptide patterns (Fig 4), indicating that the 116 kDa proteins present in both fractions are identical. Considerable amounts of the same protein were also present in the membrane protein fraction (Fig 3c).

To quantify the amounts of the 116 kDa protein present in the chromatin, membrane and soluble protein fractions, duplicate gels from those shown in Figs 2 and 3,

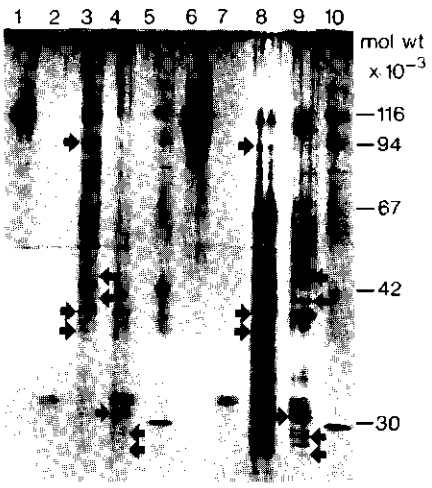


Fig. 4: Peptides cleaved by protease V8 from the 116 kDa proteins present in extracts made 240 h after inoculation, as separated in 0.1% SDS-containing 7.5-20% linear gradient polyacrylamide gel stained with silver. Lane 1: undigested 116 kDa band from chromatin; lane 2: 0.1 μg protease V8; lane 3: 116 kDa band from chromatin + 0.02 μg protease V8; lane 4: as 3, but 0.1 μg protease V8; lane 5: marker proteins; lane 6: undigested 116 kDa band from soluble protein fraction; lane 7: as lane 2; lane 8: soluble 116 kDa band + 0.02 μg protease V8; lane 9: as 8, but 0.1 μg protease V8; lane 10: marker proteins. Arrows indicate characteristic peptides produced by protease V8 action.

stained with Coomassie Blue, were scanned. From the heights of the peaks and the protein contents of the different fractions, the amounts of 116 kDa protein associated with the nucleus, cytoplasm and membranes, were calculated, assuming that all non-bound 116 kDa was present in the cytoplasm and not in vacuoles and intercellular spaces (Table 1). Around 11% of the protein was associated with the chromatin, the remainder being divided equally between the soluble and membrane fractions. From these figures, the actual concentrations of the 116 kDa protein in cytoplasm and nucleus were calculated. By analyzing light micrographs, the volumes of nucleus and cytoplasm in typical mesophyll cells of young, developing leaves could be determined. Since tobacco leaf growth at this stage, proceeds primarily through water uptake in the vacuole, no significant differences in nuclear and cytoplasmic volume ratios were observed between leaves of 4, 6 or 8 cm long. Thus, the concentration of the 116 kDa protein in nucleus and cytoplasm could be estimated directly by dividing the amount of the protein in these fractions by the average fraction volume. These calculations indicate that the concentration of the 116 kDa protein in the nucleus is about 8-fold higher than in the cytoplasm (Table 1). This clearly suggests that the 116 kDa protein is preferentially associated with the chromatin.

Table 1

Quantification of the 116 kDa protein in 4-8 cm long mosaic diseased leaves from TMV-infected Samsun tobacco.

	'Nucleus' [a]	'Cytoplasm' [b]	'Membrane' [c]	Ratio N/C/M
Protein content [d]	3.1 ± 0.2	11.2 ± 0.3	5.2 ± 0.3	1.0: 3.6: 1.7
Percentage of protein in 116 kDa band [e]	0.59	0.65	1.42	1.0: 1.1: 2.4
Amount of 116 kDa protein [f]	18.4 ± 1.0	72.6 ± 1.9	75.0 ± 4.0	1.0: 4.0: 4.1
Average volume [g]	13.4 ± 6.8	429.8 ± 222.2	n.d.	1.0: 32.1
Concentration per cell [h]	1.32 ± 0.1	0.16 ± 0.01	n.d.	8.2: 1.0

a. Protein associated with chromatin

b. Total soluble protein (Total leaf protein - chromatin-associated proteins - membrane-associated proteins)

c. Protein in *72,000 g* pellet (see Materials and Methods)

d. mg.g leaf^{-1}

e. Expressed as percentage of total scan surface occupied by the 116 kDa peak, assuming a Gaussian distribution, on gels containing equal amounts of chromatin-associated, soluble and membrane-associated proteins

f. µg.g leaf^{-1}

g. µm^3 (N=40)

h. µg.µm^{-3}.10^{-8}. The number of cells per g leaf (1.04.10^{-8}) was calculated from the average amount of DNA per g leaf [17] divided by the average amount of DNA per nucleus (10 pg, [5])

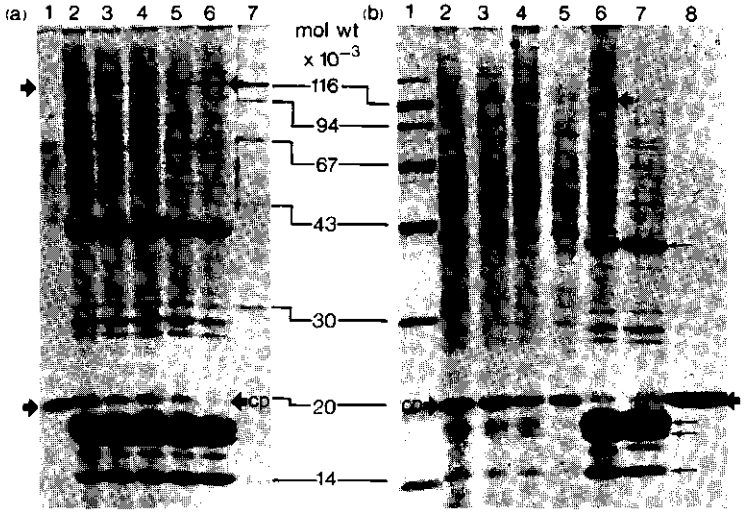


Fig. 5: Electrophoretic patterns of chromatin-associated proteins in 0.1% SDS-containing 7.5-17.5% linear gradient polyacrylamide gels stained with Coomassie Blue. Proteins were isolated from mosaic-diseased leaves of Samsun tobacco plants 240 h after inoculation with TMV. Chromatin was dissociated in (a) 10 mM Tris-HCl, 1 mM $NaHSO_3$, 1% 2-mercaptoethanol pH 8.0 and 6 M urea without NaCl (lane 1) or in the presence of 0.5 M (lane 2), 1.0 M (lane 3), 1.5 M (lane 4), 2.0 M (lane 5) or 3.0 M NaCl (lane 6) or (b) 10 mM Tris-HCl, 1 mM $NaHSO_3$, 1% 2-mercaptoethanol pH 8.0 in the presence of 6 M urea (lane 2), 7 M urea (lane 3), 8 M urea (lane 4), 9 M urea (lane 5), or 6 M urea and 3 M NaCl (lane 6). Lane 7 contains acid-soluble proteins and lane 8 purified TMV coat protein (cp). Lane 7 in (a) and 1 in (b) contain marker proteins. Arrows indicate the positions of the 116 kDa protein and TMV coat protein. Arrows in lane 7 indicate histone bands.

Effect of chromatin dissociation conditions

When chromatin was dissociated in the presence of increasing concentrations of NaCl, the patterns shown in Fig 5a were obtained. Without NaCl, only a fraction of the amount of protein normally released with 3 M NaCl was solubilized. Most of the solubilized protein consisted of a single prominent band with a molecular mass of about 20 kDa.

In the presence of 0.5 M NaCl, a pattern essentially similar to that obtained with 3 M NaCl was visible, and which included both the 116 kDa and the 20 kDa bands. However, increasing amounts of NaCl in the dissociation medium resulted in a decrease in the intensity of the 20 kDa band, leading to its virtual absence in the 3 M NaCl/6 M urea extract. When purified TMV coat protein was subjected to electrophoresis under the same conditions (Fig 5b), its position was found to coincide with that of the 20 kDa protein, thus identifying it again as TMV coat protein (18).

When chromatin was dissociated in media without NaCl but with increasing amounts of urea, and higher amounts of protein were applied to the gel, additional protein bands were revealed. As shown in Fig 5b, essentially all of the non-histone proteins were released in the presence of urea only, but only to a limited and varying extent (cf Fig. 5a). Increasing the concentration of urea from 6 M to 9 M (the concentration of urea used in the dissociation medium for 2-D gel electrophoresis) did not affect the protein pattern. However, the most prominent band was again TMV coat protein, and the 116 kDa protein and the acid-soluble histones were not solubilized under these conditions. Addition of NaCl was necessary to solubilize both the 116 kDa protein and the histones (Fig 5b, lane 6), and the resulting pattern showed additional minor differences with those obtained with urea only. The 116 kDa protein clearly differed from the histones, however, in not being released from the chromatin by 0.4 N H_2SO_4. Thus, whereas TMV coat protein was solubilized in urea, the 116 kDa protein was only released in the presence of salt.

Discussion

TMV coat protein readily dissociated from tobacco chromatin in buffers containing urea only, whereas for the solubilization of the 116 kDa protein and the histones, as well as the quantitative release of the other chromatin-associated proteins, the presence of NaCl proved to be necessary. This differential behaviour explains why in our previous experiments using 2-D gel electrophoresis (18), coat protein was found as a very prominent spot, whereas most other chromatin-associated proteins were present in relatively small amounts: since the chromatin dissociation medium used to obtain the proteins for the isoelectric focusing step did not contain NaCl, only a limited amount of the protein was solubilized, most of it being TMV coat protein. Adding NaCl to the dissociation medium substantially increased the overall yield of chromatin-associated proteins. However, the intensity of the coat protein band decreased with increasing salt concentration, until at 3 M NaCl virtually no coat protein re-

mained. Probably, due to hydrophobic interactions (2), TMV coat protein aggregated at this high salt concentration (9) and was removed with the DNA during centrifugation. This explains why coat protein was not previously observed when chromatin was dissociated in 3 M NaCl for one-dimensional gel electrophoresis (17).

From these dissociation experiments the conclusion can be drawn that the 116 kDa protein is bound more tightly to the chromatin than TMV coat protein, the need for higher salt concentrations to dissociate the protein from the chromatin pointing to a direct interaction of the protein with nucleic acid (13, 20).

Besides associated with chromatin, the 116 kDa protein was also found associated with membranes and free in the soluble protein fraction. Since it was not possible to determine the actual volume of the membranes, its concentration in the membrane fraction could not be assessed. However, the concentration of the 116 kDa protein in the nucleus appeared to be about 8-fold higher than in the cytoplasm, suggesting that it may be synthesized in the cytoplasm or on cytoplasmic membranes and transported into the nucleus. Its relatively basic isoelectric point (18) and its behaviour as a real chromatin protein in dissociation experiments, make it an interesting candidate for a regulatory protein interacting directly with the DNA and affecting gene expression. As the first time the 116 kDa protein was discernable in chromatin coincided with the appearance of the first symptoms (vein clearing), it might well play a role in induction or expression of symptoms in developing leaves. Symptoms might then arise by the combined result of altered gene expression and viral multiplication.

So far the occurrence of the 116 kDa protein was found to be confined to plants infected with TMV (18). Our present observations indicate that its accumulation is closely associated with virus multiplication. As it is known that (a) in TMV-infected plants a virus-induced RNA-dependent RNA polymerase is associated with the membrane fraction (11, 19) and (b) TMV codes for a polypeptide with similar molecular mass, appearing in the cytoplasm of infected leaves and protoplasts (6, 12), which is suggested to be part of a RNA-dependent RNA polymerase (12), it is not inconceivable that the 116 kDa protein might be virus-coded. Its presently established preferential association with chromatin would then imply that a plant virus could perturb the metabolism of its host at the genome level. This far-reaching possibility is now being further explored.

References

1. Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72, 248-254.
2. Butler, P. J. (1984). The current picture of the structure and assembly of tobacco mosaic virus. *Journal of general Virology* 65, 253-279.
3. Clark, M. F. and Adams, A. N. (1977). Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *Journal of general Virology* 34, 475-483.

4. Cleveland, D. W., Fischer, S. G., Kirschner, M. W. and Laemmli, U. K. (1979). Peptide mapping by limited proteolysis in sodium dodecylsulphate and analysis by bel electrophoresis. *Journal of Biological Chemistry* 252, 1102-1106.
5. Hamilton, R. H., Kuensch, U. and Temperli, A. (1972). Simple rapid procedures for isolation of tobacco leaf nuclei. *Analytical Biochemistry* 49, 48-57.
6. Huber, R. (1979). Proteins synthesized in tobacco mosaic virus infected protoplasts. *PhD-thesis*, Mededelingen Landbouwhogeschool, pp 1-103.
7. Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685.
8. Matthews, R. E. F. (1981). In: *Plant Virology* 2nd Edition, pp 190-209. Academic Press, New York and London.
9. Melander, W. and Horvath, C. (1977). Salt effects on hydrophobic interactions in precipitation and chromatography of proteins: an interpretation of the lyotropic series. *Archives of Biochemistry and Biophysics* 183, 200-215.
10. Morrissey, J. H. (1981). Silverstain for proteins in polyacrylamide gels.A modified procedure with enhanced uniform sensitivity. *Analytical Biochemistry* 117, 307-310.
11. Romaine, C. P. and Zaitlin, M. (1978). RNA-dependent RNA polymerases in uninfected and tobacco mosaic virus-infected tobacco leaves: Viral-induced stimulation of a host polymerase activity. *Virology* 86, 241-253.
12. Scalla, R., Romaine, P., Asselin, A., Rigaud, J. and Zaitlin, M. (1978). An *in vivo* study of a non structural polypeptide synthesized upon TMV infection and its identification with a polypeptide synthesized *in vitro* from TMV-RNA. *Virology* 91, 182-193.
13. Schumacher, J., Sänger, H. L. and Riesner, D. (1983). Subcellular localization of viroids in highly purified nuclei from tomato leaf tissue. *EMBO Journal* 2, 1549-1555.
14. Shaw, L. M. J. and Huang, R. C. C. (1970). A description of two procedures which avoid the use of extreme pH conditions for the resolution of components isolated from chromatins prepared from pig cerebellar and pituitary nuclei. *Biochemistry* 9, 4530- 4542.
15. Towill, L. E. and Noodén, L. D. (1973). An electrophoretic analysis of the acid-soluble chromosomal proteins from different organs of maize seedlings. *Plant and Cell Physiology* 14, 851-863.
16. Van Loon, L. C. and Van Kammen, A. (1970). Polyacrylamide disc electrophoresis of the soluble leaf proteins from *Nicotiana tabacum* var 'Samsun' and 'Samsun NN'. II. Changes in protein constitution after infection with tobacco mosaic virus. *Virology* 40, 199-211.
17. Van Telgen, H. J. and Van Loon, L. C. (1983). Isolation and electrophoretic analysis of chromatin-associated proteins from virus-infected tobacco leaves. *Zeitschrift für Pflanzenphysiologie* 112, 171-180.
18. Van Telgen, H. J., Van der Zaal, E. J. and Van Loon, L. C. (1984). Evidence for the association of viral coat proteins with host chromatin in mosaic diseased tobacco leaves. *Physiological Plant Pathology*, in press.
19. Zaitlin, M., Duda, C. T. and Petti, M. A. (1973). Replication of tobacco mosaic virus. V: Properties of the bound and solubilized replicase. *Virology* 53, 300-311.
20. Zieve, G. and Penman, S. (1981). Subnuclear particles containing a small nuclear RNA and heterogeneous nuclear RNA. *Journal of Molecular Biology* 145, 501-523.

Chapter 5
The Tobacco Mosaic Virus-Coded 126 kDa Polypeptide Is Associated with Host Chromatin in Mosaic-Diseased Tobacco Plants

Summary

The new 116 kDa chromatin-associated protein from mosaic-diseased leaves of tobacco mosaic virus (TMV)-infected tobacco comigrated in polyacrylamide gels with the 126 kDa translational product synthesized *in vitro* from TMV RNA. Limited proteolysis of the 116 kDa polypeptide and of the 126 kDa translational product with protease V8 generated the same peptides, indicating both proteins to be identical. Thus, the virally-coded 126 kDa protein is associated with host chromatin and may perturb host metabolism at the genome level.

Results and Discussion

Upon infection of sensitive tobacco plants with tobacco mosaic virus (TMV), young leaves of less than about 15 mm in length at the time of inoculation, start showing mosaic symptoms 7-10 days later (1-3). Concomitant with symptom appearance two additional proteins, with estimated molecular masses of 116 and 20 kilodaltons (kDa), respectively, have become discernable among the chromatin-associated proteins (4, 5). The 20 kDa protein was selectively dissociated from the chromatin in 6 M urea and identified serologically as the viral coat protein (5). However, the 116 kDa protein, which also appeared TMV-specific, remained tightly associated with the chromatin under these conditions and was only released, together with the other chromatin-associated proteins, in the presence of 0.5 M NaCl. Although the 116 kDa protein was also present both in the soluble protein and in the sedimentable membrane fractions of mosaic-diseased leaves, its concentration in the nucleus was about 8-fold higher than in the cytoplasm, suggesting it to behave as a real chromatin protein (6). Both a 126 kDa polypeptide migrating similarly to β-galactosidase (116 kDa) in SDS-polyacrylamide gels (7, 8), and its 183 kDa readthrough extension are coded by TMV (8-12) and have been identified in inoculated leaves and infected protoplasts (7, 13). Although they are assumed to play a role in viral replication (14), so far no function of these proteins has been demonstrated. We report here that the 116 kDa polypeptide associated with the chromatin in mosaic-diseased leaves and the 126 kDa product of TMV RNA are identical. The association between this virally-coded protein and the chromatin of its host suggests that this viral product may interfere directly with host gene expression, resulting in pathogenesis and symptom induction.

Tobacco plants (*Nicotiana tabacum* L. cv. Samsun) were grown in a greenhouse (4) and, when 7-8 weeks old, inoculated on two fully-grown leaves with TMV W U1 (15). When mosaic symptoms had become visible, mosaic-showing leaves of 4-8 cm in length were harvested, nuclei isolated and chromatin prepared (4). The chromatin

proteins were extracted (16) and prepared for SDS-polyacrylamide gel electrophoresis as described previously (4). TMV RNA was isolated (after 17) from purified TMV (18) by adding an equal volume of 50 mM Tris-HCl, 10 mM EDTA, pH 8.4, containing 4% (w/v) Sarkosyl-NL97, 4% (w/v) SDS and 4% (w/v) sodium tri-isopropylnaphtalene sulfonate. The mixture was heated for 2 min at 65 °C. RNA was purified by three extractions with phenol and two extractions with chloroform, respectively, and precipitated with two volumes of ethanol at −20 °C. The precipitate was washed three times with 96% ethanol, dried, and dissolved in water at a concentration of 1 $g \cdot l^{-1}$. Translation of RNA in rabbit reticulocyte lysate (a generous gift from Dr Richard Jackson, Department of Biochemistry, University of Cambridge, U.K.) was performed under the conditions described in ref. 11 and 19. ^{35}S-methionine (New England Nuclear, specific activity 1180 $Ci \cdot mmol^{-1}$) was used as radioactive amino acid (10 μCi per 10 μl reaction mixture) and incubation was at 30 °C. The endogeneous activity of the lysate used (no RNA added) was undetectable. The chromatin-associated proteins and the ^{35}S-labeled *in vitro* translation products were analyzed in linear gradient SDS-polyacrylamide gels. Prior to electrophoresis all samples were heated in a boiling waterbath in the presence of 1% SDS and 5% 2-mercaptoethanol.

As shown in Fig. 1, the new 116 kDa band present among the chromatin-asso-

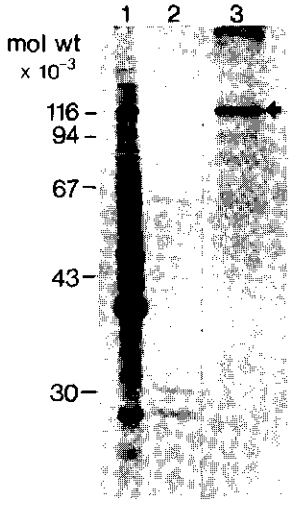


Fig. 1: Electrophoretic pattern in 10-17.5% linear gradient polyacrylamide gel containing 0.1% SDS (after ref. 27) of chromatin-associated proteins from mosaic-diseased leaves of TMV W U1 infected Samsun tobacco plants (lane 1) and of ^{35}S-labeled products obtained after *in vitro* translation of TMV RNA in rabbit reticulocyte system (lane 2), stained with Coomassie Blue. Autoradiogram of lane 2 is shown in lane 3, the arrow indicating the 126 kDa polypeptide.

ciated proteins of mosaic-diseased leaves comigrated with the 126 kDa polypeptide synthesized from TMV RNA *in vitro*. To further verify the identity of the 116 kDa protein, slices containing the 116 kDa region were cut from duplicate lanes of the gel shown in Fig. 1, and subjected to limited proteolysis by protease V8 (20).Incubation of the slices containing the 116 kDa region from mosaic-diseased leaves resulted in a specific pattern (Fig. 2a, lanes 4-6) which clearly differed from that of a control slice containing the chromatin-associated proteins in the 116 kDa region from leaves of non-infected plants (lane 2). Particularly the peptide fragments with molecular masses of 30 kDa and less appeared characteristic of the 116 kDa band from the chro-

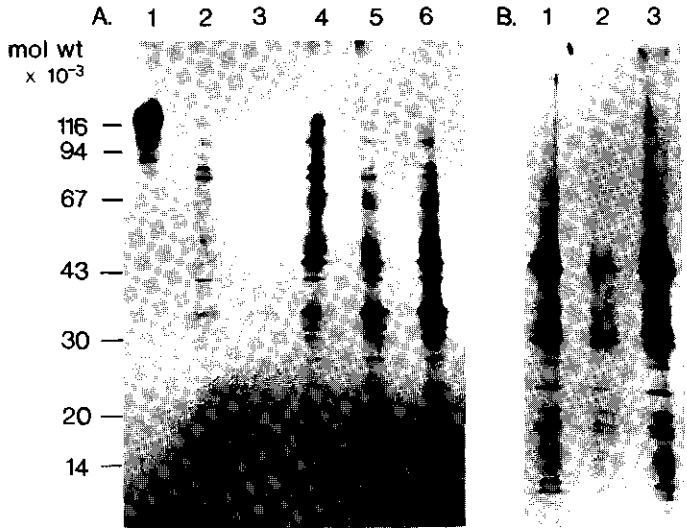


Fig. 2: Electrophoretic patterns in 7.5-20% linear gradient SDS-polyacrylamide gel (ref. 27) of peptide fragments obtained after digestion of the 116 kDa bands of Fig. 1 with protease V8. From neighbouring lanes of the gel shown in Fig. 1, slices containing the 116 kDa regions were cut and subsequently treated as described in ref. 20. Fig. 2a shows the silver-stained (ref. 28) patterns obtained from the 116 kDa regions of the chromatin-associated protein gel profiles. Lane 1: 116 kDa region from TMV-infected leaves, not treated with protease V8. Lane 2: Control slice containing the 116 kDa region from non-infected plants treated with 0.05 μg protease V8. Lane 3: contains 0.1 μg protease V8 only. Lane 4: 116 kDa region from TMV-infected leaves treated with 0.05 μg protease V8. Lane 5: 116 kDa region from TMV-infected leaves treated with 0.1 μg protease V8. Lane 6: half slice from 116 kDa region from TMV-infected leaves + half slice from 116 kDa region synthesized *in vitro* from TMV RNA treated with 0.1 μg protease V8. Fig 2b shows the autoradiogram of the pattern of ^{35}S-labeled fragments from peptides obtained by protease treatment of the 126 kDa polypeptide synthesized *in vitro* from TMV RNA. Lane 1: identical to lane 6 of Fig. 2a. Lane 2: 126 kDa region synthesized *in vitro* treated with 0.1 μg protease V8. Lane 3: 116 kDa region synthesized *in vitro* treated with 0.05 μg protease V8.

matin of mosaic-diseased leaves. The same specific peptide pattern was obtained by digestion of the ^{35}S-labeled 126 kDa band synthesized from TMV RNA *in vitro* (Fig. 2b). Thus, the 116 kDa protein found associated with chromatin in TMV mosaic-diseased tobacco leaves and the 126 kDa product synthesized *in vitro* from TMV RNA are identical.

Since upon TMV infection the 126 kDa protein is synthesized in relatively high amounts (21), it was investigated whether the virally-coded 126 kDa protein might associate preferentially with the chromatin during nuclei isolation. To this end, nuclei were isolated from non-infected 'Samsun' tobacco leaves, and the crude nuclear pellet obtained after treatment of the homogenate with Triton X-100 was resuspended in buffer containing ^{35}S-labeled 126 kDa protein synthesized *in vitro*. After incubation for 1 h, the nuclei were further purified and chromatin isolated. Under these conditions, no radioactivity was recovered among the chromatin-associated proteins upon separation in SDS-polyacrylamide gels, thus ruling out spontaneous association.

The function suggested for the TMV-coded 116 kDa protein is that of the virally-coded RNA-dependent RNA polymerase (7, 14). However, in preparations of TMV-specific RNA polymerase complexes from infected leaves (22-24) or protoplasts (13), the TMV-coded 126 kDa protein was never detected. Furthermore, the TMV RNA replication complex was found in a "membranous complex bound to cytoplasmic ribosomes" (14) and not associated with mitochondria, chloroplasts or nuclei. Moreover, very recently Kamer and Argos (unpublished results) showed by computer analysis that the 57 kDa readthrough part of the 183 kDa protein, but not the 126 kDa polypeptide, exhibits distant amino acid sequence homology to the RNA-dependent RNA polymerase from poliovirus. Thus, it seems unlikely that the 126 kDa protein associated with host chromatin in mosaic-diseased leaves has a function in viral replication. However, since the protein is also present in the cytoplasm and can associate with membranes, it may interact with different host proteins and thus provide for different functions in nucleus and cytoplasm.

In view of its relatively strong association with host chromatin and the fact that its appearance coincides with the first mosaic symptoms (6), the TMV-coded 126 kDa protein may well be involved in pathogenesis and symptom induction. Recently, it was shown that the oncogenic properties of cells, transformed by adenovirus type 12, are caused by the product of the 13 S mRNA, which is transcribed from early region 1a of the adenovirus genome. It is suggested that the polypeptide encoded for by the 13 S Ad12 E1a mRNA functions as an activator of a cellular gene via inactivation of a repressor (25). The TMV 116 kDa protein might act in a similar fashion. By binding to specific genes in the nuclear DNA of newly developing tobacco leaves, it might interfere with the synthesis of specific mRNAs, thereby causing alterations in leaf growth and development resulting in the formation of mosaic symptoms.

If the association between the virally-coded protein and host chromatin is instrumental in pathogenesis, the interaction between TMV and tobacco involves recogni-

tion at the genome level. Such a specific recognition could explain specificity in virus-host plant interaction, i.e. in some types of resistance a specific association of the virally-coded protein with host chromatin would not be possible. On the other hand, viruses coding for similar polypeptides might cause similar diseases. Indeed, Haseloff *et al* (26) recently showed that plant RNA viruses having different genomic organizations show a striking similarity in amino acid sequence of nonstructural proteins. For instance, the 126 kDa TMV protein and the proteins coded for by RNAs 1 of alfalfa mosaic virus and of brome mosaic virus (both RNA viruses with a tripartite genome) show approximately 20% amino acid sequence homology (26). This strongly suggests that, despite differences in genomic organization and host range, all three viruses produce similar proteins for interacting with their hosts. Therefore, association of a virally-coded protein with host chromatin could be a more general strategy of plant RNA viruses to exploit host cells and, consequently, cause disease.

References

1. Nilsson-Tillgren, T., Kolehmainen-Sevéus, L. and Von Wettstein, D. *Molec. Gen. Genetics* 104, 124-141 (1969).
2. Atkinson, P. H. and Matthews, R. E. F. *Virology* 40, 344-356 (1970).
3. Gianinazzi, S., Deshayes, A., Martin, C. and Vernoy, R. *Phytopathol. Z.* 88, 347-354 (1977).
4. Van Telgen, H. J. and Van Loon, L. C. *Z. Pflanzenphysiol.* 112, 171-180 (1983).
5. Van Telgen, H. J., Van Der Zaal, E. J. and Van Loon, L. C. *Physiol. Plant. Pathol.* in press (1984a).
6. Van Telgen, H. J., Van Der Zaal, E. J. and Van Loon, L. C. *Physiol. Plant. Pathol.* in press (1984b).
7. Scalla, R., Romaine, P., Asselin, A., Rigaud, J. and Zaitlin, M. *Virology* 91, 182-193 (1978).
8. Pelham, H. R. B. *Nature* 272, 469-471 (1978).
9. Beachy, R. N. and Zaitlin, M. *Virology* 81, 160-164 (1977).
10. Bruening, G., Beachy, R. N., Scalla, R. and Zaitlin, M. *Virology* 71, 498-517 (1976).
11. Pelham, H. R. B. and Jackson, R. J. *Eur. J. Biochem.* 67, 247-256 (1976).
12. Goelet, P., Lomonossoff, G. P., Butler, P. J. G., Akam, M. E., Gait, M. J. and Karn, J. *Proc. Natl. Acad. Sci. USA* 79, 5818-5822 (1982).
13. Huber, R. *PhD-thesis*, pp 1-103, Agricultural University, Wageningen (1979).
14. Matthews, R. E. F. In: *Plant Virology* 2nd Edition, pp 190-209, Academic Press New York (1981).
15. Van Loon, L. C and Van Kammen, A. *Virology* 40, 199-211 (1970).
16. Shaw, L. M. J. and Huang, R. C. C. *Biochemistry* 9, 4530-4542 (1970).
17. Davies, J. W., Verver, J., Goldbach, R. W. and Van Kammen, A. *Nucleic Acids Res.* 5, 4643-4661 (1978).
18. Hebert, T. T. *Phytopathology* 53, 362 (1963).
19. Goldbach, R. W., Schilthuis, J. C. and Rezelman, G. *Biochem. Biophys. Res. Commun.* 99, 89-94 (1981).
20. Cleveland, D. W., Fischer, S. G., Kirschner, M. W. and Laemmli, U. K. *J. Biol. Chem.* 252, 1102-1106 (1979).

21. Siegel, A., Hari, V. and Kolacz, K. *Virology* 85, 494-503 (1978).
22. Zaitlin, M., Duda, C. T and Petty, M. A. *Virology* 53, 300-311 (1973).
23. Romaine, P. and Zaitlin, M. *Virology* 86, 241-253 (1978).
24. Ralph, R. K., Bullivant, S. and Wojcik, S. J. *Virology* 43, 713-716 (1971).
25. Bernards, R., Schrier, P. I., Houweling, A., Bos, J. L., Van Der Eb, A. J., Zijlstra, M. and Melief, C. J. M. *Nature* 305, 776-779 (1983).
26. Haseloff, J., Goelet, P., Zimmern, D., Ahlquist, P., Dasgupta, R. and Kaesberg, P. *Proc. Natl. Acad. Sci. USA* 81, 4358-4363 (1984).
27. Laemmli, U. K. *Nature* 227, 680-685 (1970).
28. Morrissey, J. H. *Analyt. Biochem.* 127, 307-310 (1981).

Chapter 6
Two-Dimensional Gel Electrophoretic Analysis of Chromatin-Associated Proteins from Leaves of Different Species and Varieties of *Nicotiana*

Summary

From different *Nicotiana* species (*N. sylvestris* Spegaz. *et* Comes, *N. glutinosa* L., and *N. tabacum* L. cultivars) which differ in their sensitivity towards tobacco mosaic virus (TMV), chromatin-associated protein profiles were compared by two-dimensional polyacrylamide gel electrophoresis. After removal of DNA from dissociated chromatin preparations, the remaining chromatin-associated proteins were extracted with phenol and precipitated with ethanol. The sensitive silver staining of the gel enabled visualization of up to 350 different polypeptides.

Comparative analysis showed that over 90% of the polypeptides were similar in all *Nicotiana's*, but discrete differences provided unique patterns for each of the species and cultivars examined. However, no specific polypeptide spot could be associated with the presence of the gene *N*, which governs hypersensitivity towards TMV. Nevertheless, the method presented here allows study of the chromatin proteins involved in the regulation of gene expression during growth and development, as well as analyses of the influence of environmental factors on genetic regulatory elements.

Introduction

Tobacco (*Nicotiana tabacum*) probably originated as an amphidiploid from ancestors of the present-day species *N. sylvestris* and *N. tomentosiformis* (Valleau, 1952; Holmes, 1955). These species are all sensitive to tobacco mosaic virus (TMV) (Holmes, 1960) and, upon infection with the common (U1) strain of TMV, develop systemic mosaic symptoms on the newly emerging leaves. In contrast, *N. glutinosa*, by virtue of the presence of the nuclear resistance gene *N*, localizes TMV at the site of penetration of the virus into the leaf, forming local necrotic lesions (Holmes, 1929). By crossing *N. tabacum* and *N. glutinosa*, the *N*-gene-carrying *Hg* chromosome of *N. glutinosa* has been introduced into *N. tabacum* (Holmes, 1938; Valleau, 1952; Takahashi, 1956), yielding new tobacco cultivars that respond to TMV infection with the hypersensitive reaction characteristic of *N. glutinosa*.

Tobacco varieties lacking the *N* gene are capable of responding hypersensitively to viruses other than TMV, such as tobacco necrosis virus, and this reaction is physiologically and biochemically indistinguishable from the reaction of *N*-gene-containing tobacco cultivars to TMV. Hence, the hypersensitive reaction itself is aspecific (Van Loon, 1983) and the *N* gene functions as an element specifically controlling the interaction between tobacco and TMV. Since virus multiplication and spread in both resistant, *N*-gene-containing tobacco's and in sensitive tobacco's carrying the recessive allele *n*, are similar during at least the first 24 h after infection (Otsuki *et al*, 1972;

Konate, 1984), the resistance conferred by the *N* gene is not preformed. Indeed, the *N* gene needs to be active for a minimum of 6 h between 15 and 24 h after inoculation to initiate the sequence of events culminating in necrotic lesion formation and virus localization more than 24 h later (De Laat and Van Loon, 1983). Since the non-histone chromatin-associated proteins are considered to be the regulatory elements in specific gene expression (Thompson and Murray, 1981), the possibility was considered that the interaction between the virus and its host might be determined at the level of the chromatin-associated proteins. As was shown previously, specific changes in the chromatin-associated proteins occur after TMV infection of sensitive tobacco's (Van Telgen and Van Loon, 1983; Van Telgen *et al*, 1984a,b) and these changes consist of the presence of virally-coded proteins (Van Telgen *et al*, 1984a,c). To further elucidate the molecular basis of sensitivity to TMV in tobacco, chromatin-associated protein profiles from six *Nicotiana* species and cultivars, either possessing or lacking the *N* gene and thus differing in their reaction upon TMV infection (Table 1), were compared by two-dimensional polyacrylamide gel electrophoresis (2-DE). As compared to the procedure used previously (Van Telgen *et al*, 1984a), polypeptide resolution was considerably improved by including a phenol extraction step in the preparation of the proteins.

TABLE 1

Nicotiana species and Nicotiana tabacum cultivars possessing different genes of resistance towards TMV

Resistance gene	Present in	Reaction upon infection
N	*N. glutinosa* *N. tabacum* L. cv. Samsun NN *N. tabacum* L. cv. Xanthi-nc	Localization of all strains of TMV in necrotic lesions on inoculated leaves
N'	*N. sylvestris* *N. tabacum* L. cv. White Burley	Systemic spread of U1 strain of TMV resulting in mosaic in the leaves under 15 mm in length at the moment of inoculation. Localization of all other strains of TMV
n	*N. tabacum* L. cv. Samsun	Systemic spread of all natural strains resulting in mosaic in leaves under 15 mm in length at the moment of inoculation

Materials and methods

Plants

Tobacco plants (*Nicotiana glutinosa* L., *N. sylvestris* Spegaz. *et* Comes, and *N. tabacum* L. cvs Samsun, Samsun NN, White Burley and Xanthi-nc, were grown in a greenhouse, as described previously (Van Telgen and Van Loon, 1983). When 9-10 weeks old, the young expanding leaves (4-8 cm long) from 20 plants were harvested, nuclei isolated and chromatin prepared as described previously (Van Telgen and Van Loon, 1983).

Isolation of chromatin-associated proteins

Chromatin-associated proteins were dissociated from the DNA by resuspending the purified chromatin in 2.0 ml 10 mM Tris-HCl, 0.5 M NaCl, 6 M urea, 1 mM $NaHSO_3$, 1% (v/v) 2-mercaptoethanol, pH 8.0 (Shaw and Huang, 1970; Van Telgen *et al*, 1984b). DNA was removed by centrifugation at 100,000 *g* for 18 h.

The remaining protein-containing supernatant was extracted with an equal volume of phenol-mix (100 g distilled phenol, 0.1 g 8-hydroxyquinoline, 25 ml chloroform, and 30 ml 10 mM Tris-HCl buffer containing 1 mM $NaHSO_3$, 1% (v/v) 2-mercaptoethanol, pH 8.0) after Camacho Henriquez and Sänger (1982). The phases were separated by centrifugation for 10 min at 5,000 *g* and the aqueous phase was re-extracted with the same volume of the phenol-mix. The two phenol-phases and the interphases were pooled, and the proteins were precipitated overnight at −20 °C after addition of 5 vol of 96% ethanol. The precipitated proteins were collected by centrifugation for 10 min at 8,500 *g*, washed with 70% ethanol to remove traces of phenol, and the resulting pellet was dried under vacuum.

Preparation of chromatin-associated proteins for isoelectric focusing

The dried pellet consisting of phenol-extracted, chromatin-associated proteins, was dissolved at 25 °C in 250 μl isoelectric focusing (IEF) sample buffer (1 mM sodium-phosphate buffer pH 6.9, 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). This solution was used for IEF without further treatment.

Two-dimensional polyacrylamide gel electrophoresis

IEF was carried out in chromic acid-washed glass tubes (0.27×14 cm). To prepare 10 ml of gel mixture, 5.5 g urea was added to 1.33 ml acrylamide stock solution (28.38% (w/v) acrylamide, 1.62% (w/v) bisacrylamide), mixed with 2 ml 10% (v/v) NP-40, 0.625 ml Pharmalyte 5-8, and 1.70 ml distilled water (final acrylamide concentration: 4%). The urea was dissolved by warming the solution at 37 °C while swirling. The solution was pressed through a Millipore filter (pore size 0.3 μm),

and to the filtered solution 10 μl 10% (w/v) ammonium persulfate and 7 μl TEMED were added. The tubes were filled to a height of 12 cm, overlayed with 8 M urea, and stored at 25 °C in the dark. After polymerization was completed, the 8 M urea was replaced by sample overlay solution (4.24 g urea, 2 ml 10% (v/v) NP-40, and 0.625 ml Pharmalyte 5-8 per 10 ml), and stored until use.

The tubes were placed in an electrophoresis apparatus, filled to the edges with fresh sample overlay solution and the upper, cathodic reservoir was carefully filled with 10 mM ethanolamine. The lower, anodic reservoir contained 10 mM (DL)-glutamic acid. Using a precision syringe, 50 μl of sample containing approximately 24 μg of protein, was laid underneath the sample overlay. IEF was carried out at room temperature for 30 min at 100 V, 30 min at 200 V, subsequently 18 h at 300 V, and finally at least 2 h at 400 V. Afterwards the gels were extruded from the tubes using equilibration buffer (62.5 mM Tris-HCl, 2.3% (w/v) sodium dodecyl sulphate (SDS), 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, pH 6.9) for wetting, and equilibrated at room temperature in 15 ml equilibration buffer per gel for no longer than 15 min. After equilibration the IEF gel was placed on top of the 4% stacking gel of the second dimension, 1.5 mm thick, 0.1% SDS containing 10-15% linear gradient polyacrylamide slab gel (Laemmli, 1970), and embedded in 1% (w/v) agarose in equilibration buffer to which 0.025% (w/v) bromophenol blue (BPB) had been added as a tracking dye. Electrophoresis in the second dimension was started at a constant current of 20 mA per gel until the BPB marker had reached the separation gel. Thereupon the current was raised to 30 mA per gel and electrophoresis was continued until the BPB front had run off the gel (4.5-5 h). Molecular weight markers included β-galactosidase (116,000), ovalbumin (43,000), carbonic anhydrase (30,000), and trypsin inhibitor (20,000).

Silver staining of the second dimension gel

The second dimension gel was stained with silver essentially as described by Morrissey (1981) with two minor modifications. Fixation was carried out in 7.5% glutaraldehyde instead of 10% and, after fixation, the gel was rinsed at least three times with glass-distilled water before leaving it to soak in a large volume of water overnight. The next day washing was continued for at least 5 h, changing the distilled water every 30 min. By this procedure, background staining was substantially reduced. The reproducibility of the patterns was checked by repeating the whole procedure at least once.

Results

Preparation of chromatin-associated proteins for analysis

When purified chromatin was resuspended in lysis buffer containing 9.5 M urea as described by O'Farrell and O'Farrell (1977), only small amounts of the chromatin-

associated proteins were released from the DNA (Van Telgen *et al*, 1984b). Addition of 0.5 M NaCl to the dissociation medium resulted in a 5-fold increase of the amount of protein liberated (Van Telgen *et al*, 1984b), but extracts containing NaCl cause artefacts in IEF (O'Farrell and O'Farrell, 1977). This problem was overcome by removal of the NaCl by phenol extraction and subsequent precipitation of the proteins with ethanol, yielding the additional advantage of protein concentration. The resulting protein pellet readily dissolved in IEF sample buffer. Protein solutions thus obtained were either used immediately for IEF, or stored at −20 °C until use. Storage at −20 °C for up to six weeks did not affect the protein patterns. Furthermore, no qualitative differences were detectable between the patterns of chromatin-associated proteins prepared in this way and comparable samples obtained through direct dissociation of the chromatin in the O'Farrell's lysis buffer. Quantitatively, however, protein spots were sharper and far more intense and easily recognizable if the dissociation method described above was used.

Electrophoretic analysis of chromatin-associated proteins

One-dimensional acidic-urea or SDS polyacrylamide gel electrophoresis of the chromatin-associated proteins resulted in patterns consisting of up to 50 protein bands (Van Telgen and Van Loon, 1983) and revealed no significant differences in protein profiles between different tobacco cultivars (Van Telgen *et al*, 1984a). When using 2-DE as described above, resolution was drastically improved, yielding highly reproducible patterns revealing up to 350 polypeptide spots of varying intensity with pI's ranging from 5 to 8 and molecular massess between 130 and 15 kDa (Figs 1A-F). These results demonstrate that in leaves of *Nicotiana* species a large number of proteins is associated with the nuclear DNA. Furthermore, this large number was only revealed upon 2-DE, indicating that proteins migrating as one band in SDS polyacrylamide gel electrophoresis (and, thus, having similar sizes), may greatly differ in isoelectric point.

To compare the different protein profiles, composite maps including all the protein spots visible on the gels, were drawn from the 2-DE patterns of the six species and cultivars examined. As an example the map of *N. glutinosa* is shown in Fig. 2. Each map was divided into alphabetically-labeled quadrants, using intense protein spots present in all profiles as internal references. Specific protein spots within each quadrant were numbered and thus each polypeptide was designated by coordinates based on quadrant letter and spot number.

Comparison of the composite maps revealed a basic pattern of proteins that appear to be identical in all species and cultivars examined. For instance, the proteins in quadrant D, and likewise the series of very intense spots at the boundary between quadrants G and K, and H and L, were present in all gel profiles. More than 90% of the polypeptide spots present in the two *N. tabacum* cultivars Samsun and Samsun NN

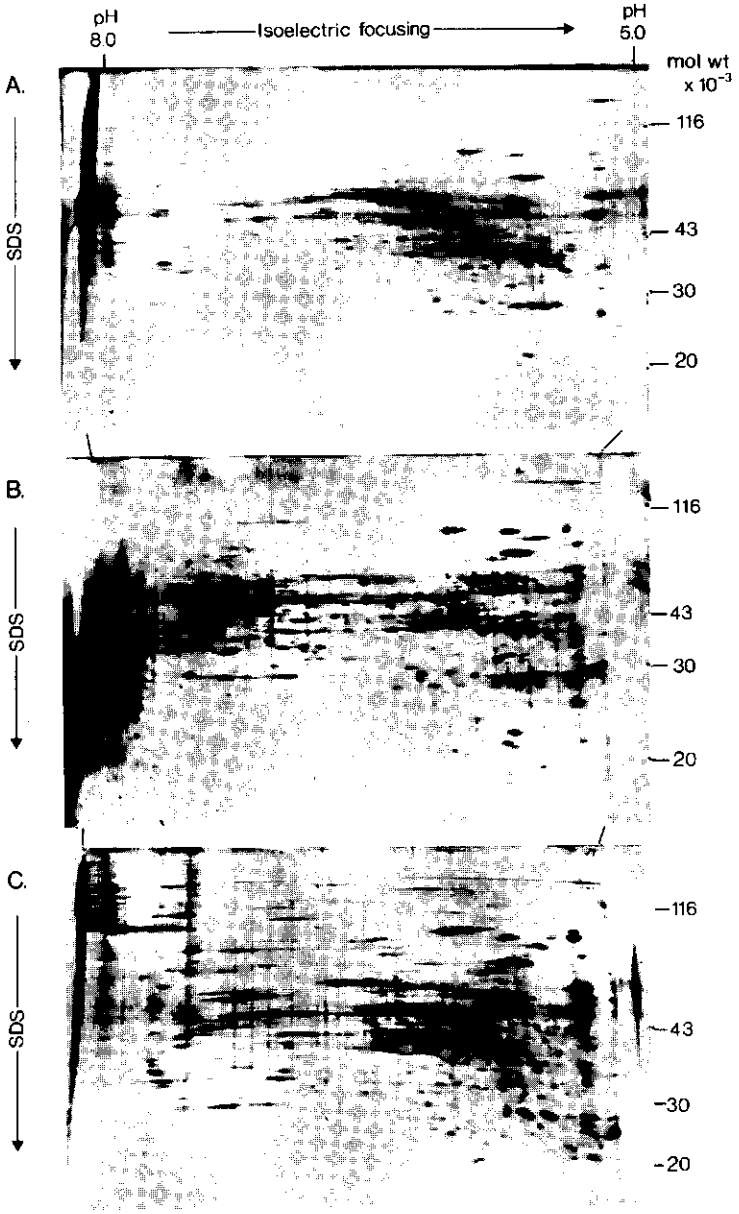
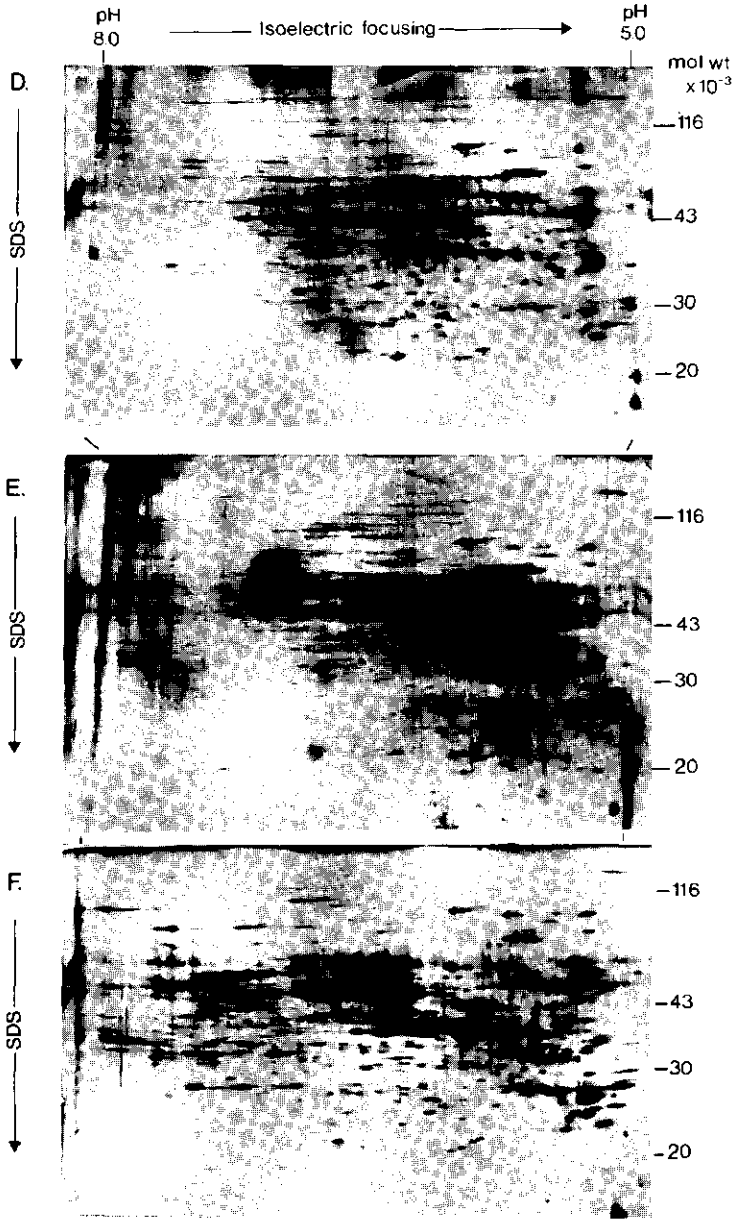


Fig. 1: Two-dimensional gel electrophoretic patterns of chromatin-associated proteins from (A) *N. tabacum* L. cv. Samsun, (B) cv. Samsun NN, (C) cv. Xanthi-nc, (D) cv. White Burley, (E) *N. sylvestris*, (F) *N. glutinosa*. IEF in the first dimension was between pH 5 and 8, and SDS electrophoresis in the second dimension was in 10-15% linear gradient polyacrylamide slab gels. 24 μg of protein was applied to the first-dimension gel. The second dimension gel slabs were stained with silver.


pH
8.0
Isoelectric focusing
pH
5.0
mol wt
x 10⁻³
D.
SDS
116
43
30
20
E.
SDS
116
43
30
20
F.
SDS
116
43
30
20

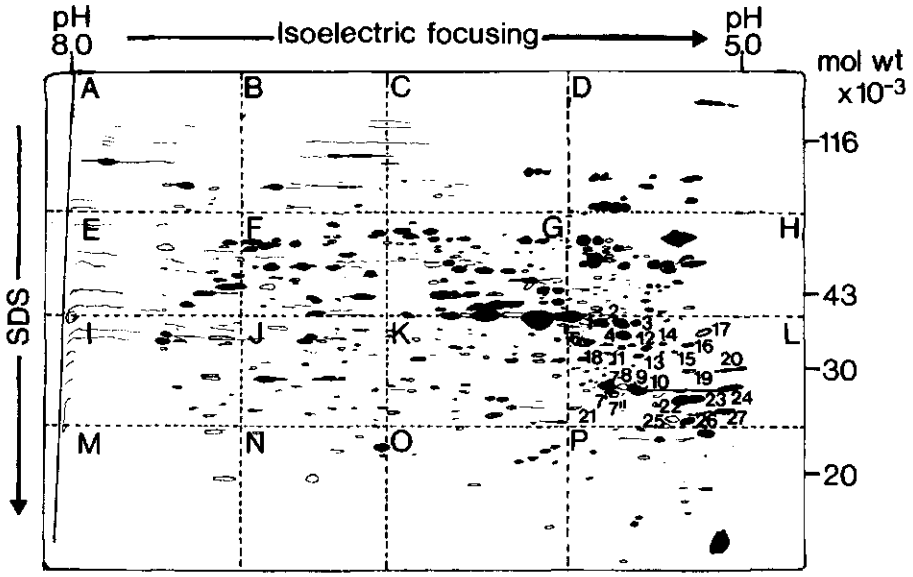


Fig. 2: Composite map of all polypeptide spots visible in the 2-DE gel of chromatin-associated proteins from *N. glutinosa* (cf. Fig. 1F). Medium-stained and faint spots are outlined, heavily stained spots are filled. Capitals indicate quadrant letter. By numbering each spot, specific polypeptides can be retraced by using coordinates based on quadrant letter and number. As an example polypeptide spots in quadrant L are numbered.

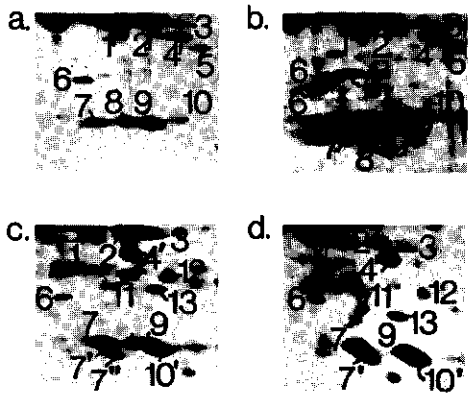


Fig. 3: Close-ups from part of quadrant L from 2-DE gels of (a) *N. tabacum* L. cv. Samsun, (b) *N. tabacum* L. cv. Samsun NN, (c) *N. glutinosa*, (d) *N. sylvestris*. Numbers define most prominent individual polypeptide spots.

were also present in the polypeptide profile of *N. glutinosa*. However, within each species or cultivar several spots discretely different in position from those in the other plants were present. As exemplified in Fig. 3, showing a magnification of part of quadrant L, all *N. tabacum* cultivars examined contained polypeptide spots L1 to L5 and L7 to L10. However, in both *N. glutinosa* and *N. sylvestris* spots L4, L5, L8 and L10 were absent, but different spots L4', L7', L7" (only in *N. glutinosa*), and L10' were present, instead. Furthermore, both in *N. sylvestris* and in *N. glutinosa* additional spots L11, L12 and L13 could be observed. Further differences can be seen in quadrants O and P (Fig 4). From spots O1, O2, O3 and O4, present in *N. glutinosa*

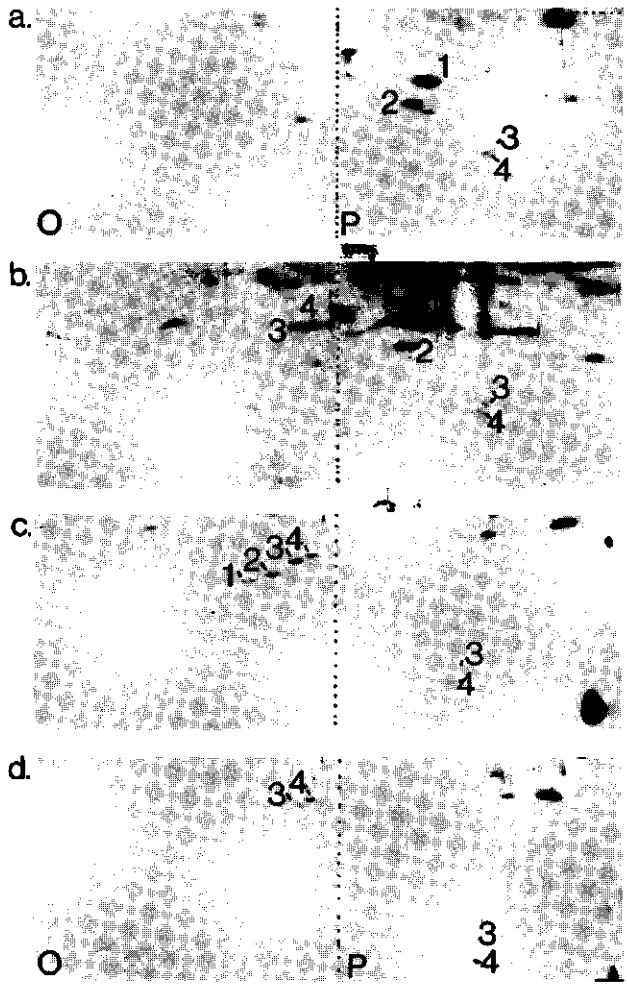


Fig. 4: Close-ups of quadrants O and P from 2-DE gels of (a) *N. tabacum* L. cv. Samsun NN, (b) *N. tabacum* L. cv. White Burley, (c) *N. glutinosa*, (d) *N. sylvestris*. Numbers are discussed in the text.

as well as in the tobacco cultivar Xanthi-nc (not shown), two spots, O1 and O2, were absent from *N. sylvestris* and tobacco cv. White Burley; only one spot, O3, was discernable in Samsun NN. Samsun similarly only showed spot O3 (not shown). In contrast, spots P1 and P2 were present in the profiles of Samsun, Samsun NN and White Burley, but absent from the profiles of Xanthi-nc and the other two *Nicotiana* species. Finally, a significant difference was observed in quadrant F (Fig 5). In five out of the six species and cultivars examined, spots F1, F2, F3, F4 and F5 were visible, but in Samsun, spot F2 was absent.

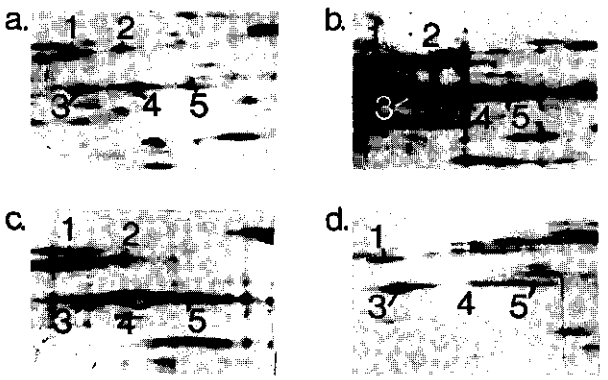


Fig. 5: Close-ups of quadrant F from 2-DE gels of (a) *N. glutinosa*, (b) *N. tabacum L. cv. Samsun NN, (c) N. sylvestris*, (d) *N. tabacum* L. cv. Samsun. Numbers are discussed in the text.

Further differences between the species and cultivars were noticeable. For instance, 90 polypeptide spots present in *N. glutinosa* were not detectable in the profiles of the tobacco cultivars Samsun NN or Samsun (Table 2). Samsun and Samsun NN showed about 90% homology. The differences, which may be ascribed to the replacement of the *H* chromosome by the *Hg* chromosome in Samsun NN, were found especially among those polypeptides which were present in small amounts.

TABLE 2

Number of polypeptide spots identical in different species of Nicotiana

	Total number of spots detected on gel	Number of spots in common with	
		N. glutinosa	*N. tabacum* cv. Samsun
N. tabacum L. cv. Samsun	281	265	281
N. tabacum L. cv. Samsun NN	281	262	236
N. glutinosa	352	352	265

However, it is difficult to ascertain whether the variation in polypeptide spots was qualitative or must be considered quantitative because similar spots were detected in some of the other tobacco's. For instance, Samsun NN differed from Samsun in 45 spots. From these 45 spots, 39 were also detected in *N. glutinosa*. Comparison with the chromatin-associated protein profile from Xanthi-nc, which also possesses the *N* gene from *N. glutinosa*, decreased the number of spots that *N*-gene-carrying tobacco's have in common to 13. However, these 13 spots were also present both in *N. sylvestris* and in White Burley, which both possess the *N'*-gene and develop mosaic symptoms upon infection with TMV U1.

DISCUSSION

By improving the procedure for preparing chromatin-associated proteins, notably by application of a phenol extraction followed by precipitation of the proteins by ethanol, it was possible to obtain highly concentrated protein samples. This high concentration of protein eliminated the need for *in vitro* radioactive labeling (Trewavas, 1979; Kuhn and Wilt, 1980) and protein detection by gel autoradiography, and enabled us to use silver staining to visualize the protein patterns. A second advantage of the improved extraction procedure is that contaminating nucleic acids are adequately removed in the phenol extraction step. Thus, one of the problems often encountered when analyzing chromatin-associated proteins by equilibrium IEF, namely 'clogging' of the basic end of the IEF gel.(O'Farrell and O'Farrell, 1977) is eliminated. Although this problem may also be circumvented by the use of non-equilibrium IEF (O'Farrell *et al*, 1977; Kuhn and Wilt, 1980), we preferred equilibrium IEF, as with this method compression of the acidic part of the pH-gradient is avoided and resolution of the relatively acidic non-histone proteins is enhanced. The highly reproducible electrophoretic profiles of the chromatin-associated proteins thus obtained were free of cytoplasmic contamination (see also Van Telgen and Van Loon, 1983), and consisted of up to 350 polypeptide spots of varying intensity. Since in a tobacco leaf approximately 20,000 genes are being transcribed (Kamalay and Goldberg, 1980), a complex pattern of proteins involved in gene regulation during leaf development is to be expected. The present study illustrates the potency of 2-DE for the analysis of chromatin-associated proteins. Using the adapted method it is possible to obtain stained protein profiles that the allow comparison of the chromatin-associated protein constitution from different plant species as well as from different organs during growth and development of one specific plant species.

Comparison of the gel electrophoretic profiles revealed a basic pattern consisting of polypeptides with apparently identical isoelectric points and molecular masses in the three species examined. The fraction of polypeptide spots common to each two species, ranged from 75-95%. This fits in with the rather close relationship between the different *Nicotiana's* examined. However, many differences were also observed, not only between species but even between cultivars within the *N. tabacum* species.

This diversity precludes interpretation of the observed differences and attribution of specific functions to certain protein spots. Differences were expected since none of the cultivars were isogenic; Samsun NN has been derived from Samsun by chromosome substitution and, in addition to the *N* gene, contains further genetic information from *N. glutinosa* (Gerstel, 1943, 1945). It can be anticipated that with plants which differ in only a single gene, it will be possible to identify specific regulatory proteins, assuming that these proteins are synthesized in amounts high enough to be detected by silver staining.

It can be concluded that each of the *Nicotiana's* examined showed a unique pattern, indicating that even closely related species and cultivars are characterized by discrete differences in their chromatin-associated protein profiles. Differences could, however, not be associated with the presence or absence of the *N*-gene that governs the hypersensitive reaction towards TMV.

References

Camacho Henriquez, A. and H. L. Sänger: Gelelectrophoretic analysis of phenol-extractable leaf proteins from different viroid-host combinations. *Arch. Virology* 74, 167-180 (1982).

De Laat, A. M. M. and L. C. van Loon: Effects of temperature, light and leaf age on ethylene production and symptom expression in virus-infected tobacco leaves. *Physiol. Pl. Pathol.* 22, 275-283 (1983).

Gerstel, D. U.: Inheritance in *Nicotiana tabacum*. XVII: Cytogenetical analysis of glutinosa-type resistance to mosaic disease. *Genetics* 28, 533-536 (1943).

Gerstel, D. U.: Inheritance in *Nicotiana tabacum*. XIX: Identification of the *tabacum* chromosome replaced by one from *N. glutinosa* in mosaic-resistent Holmes Samsun tobacco. *Genetics* 30, 448-454 (1945).

Holmes, F. O.: Local lesions in tobacco mosaic, *Bot. Gaz* 87, 39-55 (1929).

Holmes, F. O.: Inheritance of resistance to tobacco mosaic virus in tobacco, *Phytopathology* 28, 553-561 (1938).

Holmes, F. O.: Additive resistance to specific viral diseases in plants. *Ann. Appl. Biol.* 42, 129-139 (1955).

Holmes, F. O.: Inheritance in tobacco of an improved resistance to infection by tobacco mosaic virus. *Virology* 12, 59-67 (1960).

Kamalay, J. C. and R. B. Goldberg: Regulation of structural gene expression in tobacco. *Cell* 19, 935-946. (1980).

Konate, G.: Resistance des plantes a l'infection par des virus: Etude de l'etat antiviral induit par l'infection chez des tabacs hypersensibles au virus de la mosaique du tabac. *PhD-thesis*, Louis Pasteur University, Strasbourg (1984).

Kuhn, O. and F. H. Wilt: Double labeling of chromatin proteins, *in vivo* and *in vitro*, and their two-dimensional electrophoretic resolution. *Anal. Biochem.* 105, 274-280 (1980).

Laemmli, U. K.: CLeavage of structural proteins during the assembly of the head of bacteriphage T4. *Nature* 227, 680-685 (1970).

Morrissey, J. H.: Silver stain for proteins in polyacrylamide gels: A modified procedure with enhanced uniform sensitivity. *Anal. Biochem.* 117, 307-310 (1981).

O'Farrell, P. H. and P. Z. O'Farrell: Two-dimensional polyacrylamide gel electrophoretic fractionation. In: *Methods in Cell Biology* XVI, pp 407-420. G. Stein, J. Stein and L. J. Kleinsmith. (Eds). Academic Press, New York. (1977).

O'Farrell, P. Z., H. M. Goodman, P. H. O'Farrell: High resolution two-dimensional electrophoresis of basic as well as acidic proteins. *Cell* 12, 1133-1142 (1977).

Otsuki, Y., T. Shimomura and I. Takebe: Tobacco mosaic virus multiplication and expression of the *N* gene in necrotic responding tobacco varieties. *Virology* 50, 45-50 (1972).

Shaw, L. M. J. and R. C. C. Huang: A description of two procedures which avoid the use of extreme pH conditions for the resolution of components isolated from chromatins prepared from pig cerebellar and pituitary nuclei. *Biochemistry* 9, 4530-4542 (1970).

Takahashi, W. N.: Increasing the sensitivity of the local-lesion method of virus assay. *Phytopathology* 46, 654-656 (1956).

Thompson, W. F. and M. G. Murray: The nuclear genome: structure and function. In: *The biochemistry of plants*, Vol. 6: Proteins and nucleic acids, pp 1-81. A. Marcus (Ed.). Academic Press, New York (1981).

Trewavas, A. J.: Nuclear phospoproteins in germinating cereal embryos and their relationship to the control of mRNA synthesis and the onset of cell division. In: *Recent advances in the biochemistry of cereals*, pp 175-208. D. L. Laidman and R. C. Wyn Jones (Eds). Phytochemical Society Symposium (1979).

Valleau, W. D.: The evolution of susceptibility to tobacco mosaic virus in *Nicotiana* and the origin of the tobacco mosaic virus. *Phytopathology* 42, 40-42 (1952).

Van Loon, L. C.: Mechanisms of resistance in virus-infected plants. In: *The dynamics of host defence*, pp 123-190. J. A. Bailey and B. J. Deverall (Eds). Academic Press, Australia (1983).

Van Telgen, H. J. and L. C. Van Loon: Isolation and electrophoretic analysis of chromatin-associated proteins from virus-infected tobacco leaves. *Z. Pflanzenphysiol.* 112, 171-180 (1983).

Van Telgen, H. J., E. J. Van Der Zaal and L. C. Van Loon: Evidence for an association of viral coat protein with host chromatin in mosaic-diseased tobacco leaves. *Physiol. Pl. Pathol.*, in press (1984a).

Van Telgen, H. J., E. J. Van Der Zaal and L. C. Van Loon: Some characteristics of the association of the 116 kDa protein with host chromatin in tobacco leaves infected with tobacco mosaic virus. *Physiol. Pl. Pathol.*, in press (1984b).

Van Telgen, H. J., R. W. Goldbach and L. C. Van Loon: The tobacco mosaic virus-coded 126 kDa polypeptide is associated with host chromatin in mosaic-diseased tobacco plants. *Virology*, in press (1984c).

Chapter 7
General Discussion

In virus-diseased plants expression of mosaic symptoms results from interference of the virus with the regulation of genetically determined leaf development. For instance, in mottled and mosaic-diseased leaves loss of chlorophyll in the light-green or yellow areas resembles the rapid chlorophyll breakdown occurring normally in senescent leaves (Aharoni and Lieberman, 1979). However, in sensitive tobacco plants mosaic symptoms of TMV only develop on those newly emerging leaves which are not over 15 mm in length at the moment of inoculation. This mosaic pattern is laid down at a very early stage of leaf development and may remain unchanged for most of the life of the leaf (Matthews, 1981).

Light-microscopic examination of mosaic-diseased leaves has revealed that in the islands of dark green tissue (which are almost devoid of virus) chloroplasts look normal. In contrast, in the light-green or yellow areas a variety of different pathological chloroplast states can be distinguished (Matthews, 1981). Ehara and Misawa (1975) showed that in CMV-infected tobacco the induced degenerative effects change with the age at which the chloroplasts are infected. Virus infection may therefore interfere with chloroplast development. Furthermore, in mosaic-diseased leaves several enzyme activities alter similarly to those in non-infected plants after leaves have fully expanded and enter the phase of senescence, or when leaves are detached and become subject to accelerated ageing (Van Loon and Callow, 1983). These observations support the concept that induction of disease and expression of symptoms are the result of an altered regulation of leaf growth and development rather than an intervention of the virus with a single specific metabolic pathway. For this reason, virus-induced alterations were sought in the composition of those proteins that have regulatory functions in specific gene expression, the non-histone chromatin-associated proteins.

Non-histone chromatin-associated proteins were compared in healthy and systemically-infected, 4 to 8 cm long developing leaves in which mosaic symptoms had been induced by infection of lower leaves 10 days previously. Chromatin-associated protein profiles from these leaves were highly reproducible when plants were grown under identical conditions. However, the profile was clearly and specifically altered by changes in light conditions or varying leaf age, indicating that the pattern of chromatin-associated proteins does change under conditions when changes in gene expression are to be expected. For instance, under fluorescent light (no far-red) there was a strong increase in intensity of a protein band of approximately 31 kDa. Analysis of the chromatin-associated proteins from senescing leaves showed that degradation of high-molecular weight proteins had occurred. Although this may be representative of the *in vivo* situation, aspecific proteolysis may have occurred during the prolonged chromatin extraction.

Upon virus infection, changes in the chromatin-associated protein profiles were evident only when mosaic symptoms developed. These changes consisted of a limited

number of new, virus-specific proteins. Two-dimensional gel electrophoresis, further employed in order to be able to detect small changes that might have been hidden in one-dimensional SDS polyacrylamide gel electrophoresis, confirmed that neither in plants that develop systemic mosaic symptoms, nor in plants that respond with other reactions, further changes occurred. The possibility of very minute changes in these plant-virus combinations cannot be completely excluded, however. If such changes do occur, they are of a different order of magnitude compared to the changes observed in mosaic-diseased plants.

All new chromatin-associated proteins appearing in mosaic-diseased plants were identified as virus-coded proteins. The TMV-induced 116 kDa polypeptide was identical to the second-largest polypeptide coded for by the virus. The low molecular weight protein induced by TMV was serologically identified as TMV coat protein. Although the new protein bands induced by the two CMV strains co-migrated with the respective coat proteins, definite serological proof is still missing. The antisera tested reacted aspecifically with plant proteins. Attempts to purify the different CMV strains to prepare new antisera did not succeed, probably owing to instability of the virus particles.

Although the presence of coat protein in the nucleus of TMV-infected tomato leaf cells was reported before (Shalla and Amici, 1967), the experiments described above, show definitively that, in mosaic-diseased tobacco plants, viral proteins are directly associated with host chromatin and thus might interfere with leaf growth and development.

Arguments both for and against a role of coat protein in symptom expression have been put forward. Jockusch and Jockusch (1968) reported that among four closely related TMV mutants, there was an inverse correlation between the amount of intact virus particles produced and symptom severity. Disease symptoms were most severe (yellow mosaic) in plants where the greatest amounts of defective, insoluble coat protein were produced. Roberts and Wood (1981) showed that in tobacco plants infected with CMV the severe yellow mosaic-inducing P6-strain of CMV synthesized considerably more coat protein than the mild W-strain. However, studies with nitrous acid mutants of TMV (Hennig and Wittmann, 1972) indicate that TMV coat protein need not be involved in symptom expression. Mutants which possessed coat protein identical to that of wild type TMV strain U1, induced totally different symptoms. Moreover, Habili and Francki (1974) constructed a pseudo-recombinant of CMV with a different cucumo virus, tomato aspermy virus (TAV). Viruses of the cucumo virus group possess a divided genome, consisting of two large RNA molecules (RNA-1 and RNA-2) and a small RNA molecule (RNA-3) that codes for coat protein. All three components are required for infectivity. However, it is possible to replace one of the RNAs by the equivalent from another member of the same virus group (heterologous combination), rendering a pseudo-recombinant with genetic information of two different viruses. With this method genes can be localized on specific RNAs

(e.g. De Jager, 1978). In the experiments mentioned above, RNA-3 of CMV was combined with RNA's 1 and 2 from TAV. In infected plants virions encapsidated in CMV coat protein were synthesized but symptom type was that of TAV.

From the base sequence of TMV (Goelet *et al*, 1982) it can be estimated that the 'local lesion gene' defined by Kado and Knight (1966) must be approximately 1000 bases upstream of the coat protein gene, again suggesting that coat protein is not involved in symptom expression. In accordance with this concept, we found that TMV coat protein was only loosely associated with the chromatin. Dissociation was readily achieved using media with urea only. CMV coat protein is more basic than TMV coat protein (A. Maule, personal communication) and might be associated more tightly with the host chromatin. However, no further information on this point was obtained.

The site of the local lesion gene corresponds more with the reading frame of the 30 kDa protein of TMV. Leonard and Zaitlin (1982) suggested that this protein is involved in cell-to-cell transport of the virus. Although Ooshika *et al* (1984) presented serological evidence for its presence in chloroplasts, we did not detect it either associated with chromatin or among the soluble proteins from whole leaf.

In TMV-infected tobacco, the only possible candidate left for a specific symptom-inducing function seems to be the virus-coded 116 kDa polypeptide, even though the location of its gene at the 5'-end of the TMV RNA (Goelet *et al*, 1982) can not correspond with the site of the 'local lesion gene' either. The 116 kDa protein was also present among the soluble proteins and associated with membranes, but its concentration in the nucleus was calculated to be approximately 8-fold higher than in the cytoplasm. Its concentration in membranes could not be calculated but may locally exceed that in the nucleus, indicating that there may be two subcellular sites at which this protein accumulates. Nevertheless, it does behave as a real chromatin protein since it could only be dissociated from the chromatin, together with all other histone and non-histone chromatin proteins, in media containing NaCl. This is indicative of a relatively tight association with chromatin, probably through a direct protein-nucleic acid interaction (Zieve and Penman, 1981; Schumacher *et al*, 1983).

The function originally suggested for the TMV-coded 116 kDa translational product, is that of the viral contribution to the RNA-dependent RNA polymerase needed for viral replication (Scalla *et al*, 1978). TMV RNA was reported to be present in the nucleus (Reddi, 1966). In the present investigation, however, viral RNA was not resolved when nucleic acids extracted from nuclei of mosaic-diseased tobacco leaves were analyzed on gels (Van Telgen and Van Loon, 1983). Nevertheless, purified nuclei were still slightly infectious to *Nicotiana glutinosa*. Infectiosity was less than 1% of the total infectiosity present in mosaic-diseased leaves (unpublished results). On the other hand, the presumed TMV-RNA replication complex has not been found associated with nuclei, chloroplasts or mitochondria, but in a membranous complex bound to cytoplasmic ribosomes (Ralph *et al*, 1971; Zaitlin *et al*, 1973). Moreover,

Huber (1979) could not detect the 116 kDa polypeptide in partially purified RNA polymerase from TMV-infected protoplasts. Furthermore, Kamer and Argos (unpublished results) recently reported that a distant amino acid homology exists between the RNA polymerase from poliovirus and the readthrough part of the TMV-coded 183 kDa protein but no homology with the 116 kDa TMV-coded polypeptide. This could mean that the TMV-coded 116 kDa polypeptide itself is not part of the virus-specific RNA-dependent RNA polymerase, although its association with cytoplasmic membranes is suggestive of this. Possibly, the 116 kDa polypeptide associated with chromatin functions differently from the 116 kDa polypeptide associated with membrane structures.

Like Hirai and Wildman (1967) we observed that application of actinomycin D to young tobacco plants results in vein-clearing and mosaic in developing leaves (unpublished results). These observations strongly suggest that mosaic symptoms may result from a general inhibition of DNA-dependent RNA synthesis. Thus, the TMV-coded 116 kDa polypeptide might act as a repressor of host genes. Such action is compatible with the relatively high amount of 116 kDa protein present among chromatin-associated proteins in mosaic-diseased leaves. However, in the inoculated leaves, that are already fully-grown at the time of inoculation, no symptoms develop. Nevertheless, these leaves abound with virus and the 116 kDa protein is associated with the chromatin in these leaves, although its concentration turned out to be lower than in the mosaic-diseased leaves. Since upon TMV infection mosaic symptoms only develop in leaves that are not over approximately 15 mm in length at the moment of inoculation, this suggests that the 116 kDa protein might act as a repressor of genes that specifically function early in leaf development. If the 116 kDa protein associates with the chromatin in leaves where these genes have already been transcribed, no symptoms would develop.

Due to the microscopic character of the mosaic in systemically-infected leaves with its abundance of small, light-green spots among the stretches of dark-green islands, it was not possible to collect dark-green tissue free of light-green material. For that reason it was not possible to investigate whether the 116 kDa polypeptide is present in both the light-green and the dark green patches of tissue. Possibly the use of the *Lis*-a isolate of TMV, which induces a mosaic consisting of well-separated large patches of dark green and light green tissue (Whenham *et al*, 1984), may bring the answer to this question.

At temperatures above 28 °C, Samsun NN no longer reacts hypersensitively to infection with TMV, but shows a systemic spread of virus and develops mosaic symptoms, as Samsun plants do at moderate temperatures. The 116 kDa polypeptide was clearly present among the chromatin-associated proteins of Samsun plants, when these exhibited mosaic symptoms at 30 °C. It is thus surprising that this polypeptide, while present in the soluble fraction, was not discernable in the chromatin of mosaic-diseased leaves of TMV-infected Samsun NN plants grown at 30 °C.

Therefore, at this temperature the concentration of the 116 kDa polypeptide in nuclei of leaves from Samsun NN must be much lower than in Samsun. If the protein is required for symptom induction, although it may not be detectable in polyacrylamide gels, it might still be functioning, as for a regulatory protein only very few molecules per cell may suffice to block specific functions (Lin and Riggs, 1975). This might also apply to CMV-infected tobacco plants, where no protein additional to viral coat was found associated with the chromatin. Analysis of chromatin-associated proteins from virus-infected protoplasts labeled with radioactive precursors might perhaps elucidate this point. However, protoplasts do not develop symptoms. Since the stage of leaf development and the time of testing affect the rate of accumulation of the chromatin-associated 116 kDa polypeptide, it may well be that the interaction in protoplasts will be different, and thus the information obtained from protoplasts will be of limited value. To be able to support extensive virus multiplication, protoplasts have to be isolated from fully-grown leaves. Moreover, isolation of protoplasts from young, developing tobacco leaves proved technically impossible.

Recently, it was found that the TMV-coded 116 kDa polypeptide has approximately 20-30% amino acid homology with similarly-sized translational products of two other, serologically unrelated plant viruses, namely alfalfa mosaic virus and brome mosaic virus (Haseloff *et al*, 1984). Although amino acid homology does not imply functional homology, this nevertheless suggests that other plant viruses may have similar polypeptides for interacting with their hosts. It would be interesting to localize and sequence the site(s) of association of the 116 kDa polypeptide with the tobacco chromatin. Comparison with other virus-host combinations developing mosaic symptoms might then give a clue as to whether plant viruses use similar strategies to interact with their host. It would bring us a step nearer to the unraveling of symptom induction which is of great importance, because of the high losses viruses cause in economically important crops. Moreover, it may also be of importance to plant physiologists. Since symptoms may result from interference of viruses with the regulation of growth and development at the genome level, and many symptoms resemble aberrations in hormonal regulation, pathogenesis and symptom development may offer opportunities for unraveling the mode of action of plant growth regulators at the genome level. Studies of abnormalities have often given clues about the mechanisms of the processes operating in normal growth and development.

References

Aharoni, N. and Lieberman, M. (1979). Ethylene as a regulator of senescence in tobacco leaf discs. *Plant Physiol.* 64, 801-804.

De Jager, C. P. (1978). Genetische analyse van cowpea-mozaiekvirusmutanten. *PhD-thesis*, Agricultural University, Wageningen.

Ehara, Y. and Misawa, T. (1975). Occurrence of abnormal chloroplasts in tobacco leaves infected systemically with the ordinary strain of cucumber mosaic virus. *Phytopathol. Z.* 84, 233-252.

Goelet, P., Lomonossof, G. P., Butler, P. J. G., Akam, M. E., Gait, M. J. and Karn, J. (1982). Nucleotide sequence of tobacco mosaic virus RNA. *Proc. Natl. Acad. Sci. USA* 79, 5818-5822.

Habili, N. and Francki, R. I. B. (1974). Comparative studies on tomato aspermy and cucumber mosaic viruses. III. Further studies on relationship and construction of a virus from parts of two viral genomes. *Virology* 61, 443-449.

Haseloff, J., Goelet, P., Zimmern, D., Ahlquist, P., Dasgupta, R. and Kaesberg, P. (1984). Striking similarities in amino acid sequence between nonstructural proteins encoded by RNA viruses with dissimilar genomic organisation. *Proc. Natl. Acad. Sci. USA* 81, 4358-4363.

Hennig, B. and Wittman, H. G. (1972). Tobacco mosaic virus: Mutants and strains. In: *Principles and techniques in plant virology*, pp. 546-594. Ed. by C. I. Kado and H. O. Agrawal. Van Nostrand-Reinhold, New York.

Hirai, A. and Wildman, S. G. (1967). Similarity in symptoms produced in tobacco plants by Actinomycin D and TMV. *Virology* 31, 721-722.

Huber, R. (1979). Proteins synthesized in tobacco mosaic virus infected protoplasts. *PhD-thesis*, Agricultural University, Wageningen.

Jockusch, H. and Jockusch, B. (1968). Early cell death caused by mutants with defective coat protein. *Mol. Gen. Genet.* 102, 204-209.

Kado, C. I. and Knight, C. A. (1966). Location of a local lesion gene in tobacco mosaic virus RNA. *Proc. Natl. Acad. Sci. USA* 55, 1276-1283.

Leonard, D. A. and Zaitlin, M. (1982). A temperature-sensitive strain of tobacco mosaic virus defective in cell-to-cell movement generates an altered viral-coded protein. *Virology* 117, 416-424.

Lin, S. and Riggs, A. D. (1975). The general affinity of *lac* repressor for E. coli DNA: Implications for gene regulation in procaryotes and eucaryotes. *Cell* 4, 107-111.

Matthews, R. E. F. (1981). In: *Plant Virology*, 2nd Edition. Academic Press, New York and London.

Ooshika, I., Watanabe, Y., Meshi, T., Okada, Y., Igano, K., Inouye, K. and Yoshida, N. (1984). Identification of the 30K protein of TMV by immunoprecipitaion with antibodies directed against a synthetic peptide. *Virology* 132, 71-78.

Ralph, R. K., Bullivant, S. and Wojcik, S. J. (1971). Cytoplasmic membranes, a possible site of tobacco mosaic virus RNA replication. *Virology* 43, 713-716.

Reddi, K. K. (1966). Studies on the formation of tobacco mosaic virus ribonucleic acid. VII: Fate of tobacco mosaic virus after entering the host cell. *Proc. Natl. Acad. Sci. USA* 55, 593-598.

Roberts, P. L. and Wood, K. R. (1981). Comparison of protein synthesis in tobacco systemically infected with a severe or a mild strain of cucmber mosaic virus. *Phytopath.Z.* 102, 257-265.

Scalla, R., Romaine, P., Asselin, A., Rigaud, J. and Zaitlin, M. (1978). An *in vivo* study of a nonstructural polypeptide synthesized upon TMV infection and its identification with a polypeptide synthesized *in vitro* from TMV RNA. *Virology* 91, 182-193.

Schumacher, J., Sänger, H. L. and Riesner, D. (1983). Subcellular localization of viroids in highly purified nuclei from tomato leaf tissue. *EMBO-Journal* 2, 1549-1555.

Shalla, T. A. and Amici, A. (1967). The distribution of viral antigen in cells infected with tobacco mosaic virus as revealed by electron microscopy. *Virology* 31, 78-91.

Van Loon, L. C. and Callow, J. A. (1983). Transcription and translation in the diseased plant. In: *Biochemical Plant Pathology*. Ed. by J. A. Callow. Wiley & Sons Ltd, Chichester.

Van Telgen, H.J. and Van Loon, L.C. (1983). Isolation and electrophoretic analysis of chromatin-associated proteins from virus-infected tobacco leaves. *Z. Pflanzenphysiologie* 112, 171-180.

Whenham, R. J., Fraser, R. S. S. and Snow, A. (1984). Tobacco mosaic virus-induced increase in abscisic acid concentration in tobacco leaves: intracellular location and relationship to symptom severity and to extend of virus multiplication. *Physiol.Pl.Pathol.*, in press.

Zaitlin, M., Duda, C. T. and Petti, M. A. (1973). Replication of tobacco mosaic virus. V. Properties of the bound and solubilized replicase. *Virology* 53, 300-311.

Zieve, G. and Penman, S. (1981). Subnuclear particles containing a small nuclear RNA and heterogeneous nuclear RNA. *J. Mol. Biol.* 145, 501-523.

Summary

Symptoms of viral infections in plants often resemble disturbances in growth and development. Therefore, symptoms appear to result from an interference of the virus with the regulation of growth and development of the host plant. Particularly the non-histone chromatin-associated proteins are considered to be the regulators of specific gene expression. The aim of the present study was to elucidate whether upon infection of a plant with a virus, alterations occur in the non-histone chromatin-associated protein composition of the leaves.

A survey of the literature on viral pathogenesis in plants, the properties of chromatin-associated proteins, and their possible role in the regulation of specific gene expression is given in Chapter 1.

In Chapter 2 we looked for changes in the chromatin-associated protein composition of leaves from virus-infected tobacco plants. As a model system the combination 'Samsun' tobacco – tobacco mosaic virus (TMV) was used. In this combination, mosaic symptoms develop in the newly emerging leaves, the mosaic consisting of alternating patches of light-green and dark-green tissue. To ensure recovery of representative amounts of nuclei from the whole leaf and not predominantly from either light green or dark green tissue, known procedures for the isolation of nuclei had to be modified. By homogenizing the leaves in a large volume of buffer and repeated grinding of the homogenate, up to 45% of the nuclei present in the leaves were freed from the cell debris. From these nuclei chromatin was purified, and chromatin-associated proteins were dissociated from the DNA in buffer containing urea and high salt. Analysis of these proteins in SDS-containing polyacrylamide gels revealed a single consistent alteration upon TMV infection, being the induction of a new protein of about 116 kDa. The use of two-dimensional polyacrylamide gel electrophoresis showed a second alteration to occur. This second change consisted of the appearance of a new protein of about 20 kDa. This protein was serologically identified as TMV coat protein.

Further investigations into the effects of both mosaic-inducing and necrotic symptoms-producing viruses on the chromatin-associated protein constitution of different tobacco cultivars, revealed that of the viruses tested only cucumber mosaic virus (CMV) induced any specific alterations. A 'green' isolate induced a single new protein of about 28 kDa, and the 'yellow' strain P6 one of about 29 kDa. These new proteins co-migrated in SDS-containing polyacrylamide gels with the coat proteins of the respective CMV strains. CMV was also the only virus – apart from TMV – that induced mosaic symptoms. In combinations resulting in localized or systemic necrosis, no changes were observed. Thus it turned out that only if virus infection results in the development of systemic mosaic symptoms, discrete changes in the chromatin-associated protein profile occur (Chapter 3).

The presence of viral coat protein associated with host chromatin represents virus-specificity of the induced change. The presence of the 116 kDa protein exclusively

after TMV infection was also connected to the virus. It was not induced in uninfected plants during senescence of fully-grown leaves or upon ageing of detached leaves (Chapter 3). Since a primary role for coat protein in the induction of symptoms is unlikely, we further concentrated on the TMV-induced 116 kDa protein.

Whereas TMV was detected in upper leaves that become infected systemically already 96 h after inoculation of lower, fully-expanded leaves, the 116 kDa protein became discernable in the systemically-infected leaves only between 120 to 144 h after inoculation. This moment coincided with the appearance of visible symptoms (vein clearing). At that time the 116 kDa protein was also detectable, both in the soluble protein and in the sedimentable membrane fractions. However, the 116 kDa protein was found to be preferentially associated with, on the one hand, the membranous fraction and, on the other hand, the chromatin. Based on the amount of the protein in the different fractions and on morphometric analysis of tissue sections, it was calculated that the concentration in the chromatin was about eight-fold higher than in the cytoplasm. Moreover, in contrast to TMV coat protein, its dissociation from chromatin required sodium chloride. This indicates that the 116 kDa protein is bound more tightly to the chromatin than TMV coat protein. These observations strongly suggest that the 116 kDa protein may play a regulatory role in gene expression, analogous to the non-histone chromatin proteins (Chapter 4).

Since TMV is known to code for a protein of a similar molecular mass, it was investigated whether the new 116 kDa chromatin-associated protein from mosaic-diseased tobacco leaves is identical with the TMV-coded 126 kDa protein. In SDS-containing polyacrylamide gels the 116 kDa protein comigrated with the 126 kDa translational product synthesized *in vitro* from TMV RNA. Furthermore, limited digestion of the 116 kDa polypeptide and the 126 kDa translational product with protease V8 yielded the same peptide fragments, indicating that both proteins are identical (Chapter 5).

In Chapter 6 two-dimensional gel electrophoretic patterns of chromatin-associated proteins from cultivars and species of *Nicotiana* with different genetic constitutions were compared, using an improved protein preparation procedure. Notably the application of phenol extraction, followed by precipitation of proteins with ethanol, yielded highly concentrated protein samples. This procedure resulted in sharper and more intense protein spots on the gels than before. In all *Nicotiana* species and cultivars examined, 90% of the chromatin-associated proteins appeared identical. Among the 10% differing polypeptides no specific polypeptide spot(s) could be associated with the presence of the gene *N*, that governs hypersensitivity towards TMV.

Chapter 7 discusses the possible role of the 116 kDa protein in symptom development. It is proposed that the 116 kDa polypeptide may act as a repressor of plant genes that specifically function early in leaf development. This hypothesis is based on the observation that inhibition of DNA-dependent RNA synthesis with actinomycin D induces vein clearing and mosaic in developing leaves similar to the symptoms in-

duced by TMV in leaves not over 15 mm in length at the moment of inoculation. Recent results by Haseloff *et al.* (Proc. Natl. Acad. Sci. USA *81*, 4358-4363 (1984)) indicate that two similarly-sized translational products of the serologically unrelated plant viruses alfalfa mosaic virus and brome mosaic virus have approximately 20-30% amino acid homology with the TMV-coded 116 kDa polypeptide. Such observations suggest that other plant viruses may use similar strategies to interact with their hosts.

Samenvatting

In deze Nederlandse samenvatting zal worden geprobeerd de inhoud van het proefschrift zo te beschrijven dat deze ook voor niet-ingewijden begrijpelijk zal zijn. Lezers die met de beschreven materie vertrouwd zijn, zullen veelal de Engelse samenvatting kunnen begrijpen en kunnen daarin dan ook de meer in vaktaal weergegeven inhoud terugvinden.

Na binnendringen in een plant kan een virus zich sterk vermeerderen en door de plant verspreiden (systemische infectie) of de vermeerdering en verspreiding kan beperkt blijven tot een klein gebiedje rond de plaats van binnendringen (lokale infectie). Soms kunnen de cellen rondom de plaats van binnendringen zeer snel afsterven en vormt zich een necrotische lesie. In dat geval spreekt men ook wel van een overgevoeligheids- of hypersensitieve reactie. De hypersensitieve reactie is voor de praktijk van groot belang, omdat het virus effectief gelocaliseerd wordt en bovendien de planten een zekere resistentie tegen volgende virusinfecties ontwikkelen (veldresistentie). Of een virus zich na infectie al dan niet kan verspreiden, is afhankelijk van de erfelijke eigenschappen van zowel de plant als het virus. Een algemene eigenschap van veel plantevirussen is dat zij zich kunnen verspreiden in de ene gastheer, maar worden gelokaliseerd in een andere. Omgekeerd kan in één waardplant het ene virus zich wel verspreiden, terwijl andere virussen gelokaliseerd worden. Het vermogen van planten tot lokalisatie van een virus lijkt in het algemeen gereguleerd te worden door een klein aantal erfelijke factoren (genen), waarvan de uiting onder invloed van bepaalde virussen verhinderd kan worden. De resistentie wordt daardoor doorbroken. Dit impliceert dat plantevirussen in hun erfelijk materiaal informatie bezitten die hen in staat stelt lokalisering door de gastheer te vermijden, en voorts dat planten in principe het vermogen bezitten zowel om infecterende virussen te lokaliseren als om te reageren met de vorming van systemische symptomen. Gewoonlijk heeft infectie van een bepaalde plantesoort met verschillende virussen ook verschillende symptomen tot gevolg. Tevens echter kan een en hetzelfde virus verschillende symptomen teweeg brengen op verschillende plantesoorten. Symptomen in viruszieke planten zijn dan ook karakteristiek voor de specifieke combinatie van virus en gastheer. Deze symptomen kunnen zeer divers van aard zijn en varieren van nauwelijks waarneembare vlekjes op de bladeren tot zeer ernstige groeiafwijkingen. Aangezien veel van de symptomen in viruszieke planten duiden op verstoringen van de normale groei en ontwikkeling, lijkt het mogelijk dat symptomen een gevolg zijn van een interferentie van het virus met de mechanismen die groei en ontwikkeling reguleren.

De regulatie van de expressie van de specifieke genen die betrokken zijn bij de groei en ontwikkeling van de plant, vindt plaats in de kern van de plantecel. Daar ligt in de chromosomen de totale erfelijke informatie van de plant in de vorm van desoxyribonucleinezuur (DNA) opgeslagen, waarvan in elke cel slechts een specifiek deel wordt afgelezen. Veranderingen in regulatie, die door het virus worden veroorzaakt,

moeten plaatsvinden in de celkern en daar de aflezing van de plantegenen beinvloeden.

Echter, plantevirussen bezitten slechts een beperkte hoeveelheid erfelijke informatie. De meeste plantevirussen bezitten als drager van de erfelijke eigenschappen zgn. ribonucleinezuur (RNA). Naast informatie voor een manteleiwit dat in het intacte virusdeeltje het RNA tegen afbraak beschermt, bevinden zich op het RNA nog enkele genen die coderen voor specifieke eiwitten welke een succesvolle vermeerdering van het virus in de gastheer moeten garanderen. Voorts kunnen er nog genen aanwezig zijn die benodigd zijn voor overdracht door insecten of verspreiding binnen de plant. Daarmee is echter de coderingscapaciteit min of meer uitgeput, zodat er verder geen ruimte meer over is voor specifieke, symptoom-inducerende eiwitten.

Het lijkt dan ook waarschijnlijk dat symptomen een gevolg zijn van veranderingen in aard en/of hoeveelheid van die eiwitten uit de plant welke direct betrokken zijn bij de regulatie van de specifieke genexpressie in de plant. Algemeen wordt aangenomen dat van de eiwitten die geassocieerd met het chromosomale DNA het chromatine vormen, vooral de zogenaamde niet-histon-chromatine-eiwitten een regulerende functie hebben. Het in dit proefschrift beschreven onderzoek was er dan ook voornamelijk op gericht inzicht te verkrijgen of na virusinfectie veranderingen optreden in de samenstelling van deze chromatine-eiwitten en, zo ja, deze veranderingen te karakteriseren. Als modelsysteem werd daarbij de combinatie 'Samsun' tabak – tabaksmozaiekvirus (TMV) gebruikt. Na infectie van volgroeide bladeren van tabaksplanten van deze varieteit met TMV vormen zich vanaf een week na infectie mozaieksymptomen in de zich ontwikkelende bladeren. Dit mozaiek is opgebouwd uit afwisselende gebiedjes lichtgroen en donkergroen bladweefsel.

Allereerst moesten bestaande kernisolatiemethoden zo veranderd worden dat voldoende en representatieve hoeveelheden van de chromatine eiwitten in handen gekregen konden worden. Dit was noodzakelijk om er zeker van te zijn dat de geisoleerde kernen een goede weerspiegeling zouden zijn van alle kernen die in een mozaiekziek blad aanwezig zijn en niet van voornamelijk de lichtgroene of donkergroene weefseldelen. Door bladeren te vermalen in een ruime hoeveelheid buffervloeistof en het homogenaat meerdere malen te extraheren met buffer, konden grote hoeveelheden kernen uit het netwerk van celwandresten bevrijd worden. Uit de celkernen werd chromatine gezuiverd, waaruit vervolgens de chromatine eiwitten geisoleerd konden worden. Daarna werden deze eiwitten gescheiden in gels van polyacrylamide, door kleuring zichtbaar gemaakt en geanalyseerd. Hieruit bleek dat na TMV infectie in de mozaiekzieke blaadjes een nieuw eiwit met een molecuulmassa van ongeveer 116 kilodaltons (kDa) werd geinduceerd. Dit was de enige verandering die bij gebruikmaking van deze methode kon worden vastgesteld (Hoofdstuk 1). Met behulp van iets verfijndere elektroforesetechnieken (gradientgels en twee-dimensionale elektroforese) werd onderzocht of er naast het nieuwe 116 kDa-eiwit nog meer veranderingen plaatsvonden. Er werd inderdaad nog een tweede nieuw eiwit van ongeveer 20 kDa

gevonden. Dit kon serologisch worden geidentificeerd als het TMV-manteleiwit. Voorts werden naast andere virussen die mozaieksymptomen induceren ook virussen die necrotische symptomen veroorzaken in het onderzoek betrokken. Naast TMV was van alle gebruikte virussen alleen komkommermozaiekvirus (CMV) in staat discrete veranderingen in het chromatine-eiwitprofiel te induceren. Tevens was CMV het enige virus dat, net als TMV, in tabak mozaieksymptomen veroorzaakte. Na infectie met een zogenaamd 'groen' isolaat van CMV werd een nieuw eiwit van 28 kDa geinduceerd, terwijl de 'gele' stam een nieuw eiwit van 29 kDa induceerde. Door vergelijking met het eiwit uit virusdeeltjes werden aanwijzingen verkregen dat het ook hierbij ging om de manteleiwitten van de respectievelijke komkommermozaiekvirusstammen. Na infectie met virussen die necrose induceerden werden geen veranderingen waargenomen. Hieruit blijkt dat alleen wanneer zich na infectie systemische mozaieksymptomen ontwikkelen, discrete veranderingen in het eiwitprofiel optraden (Hoofdstuk 2).

De aanwezigheid van viraal manteleiwit in de kern betekent dat het om virusspecifieke veranderingen gaat. Het verschijnen van het 116 kDa eiwit uitsluitend na infectie met TMV leek ook ten nauwste samen te hangen met het virus. Dit eiwit werd niet geinduceerd in ongeinfecteerde tabaksplanten bij natuurlijke of kunstmatige veroudering waarbij, net als na virusinfectie, het blad door chlorofylverlies lichtgroen kleurt (Hoofdstuk 3).

Aangezien uit literatuurgegevens is af te leiden dat manteleiwit waarschijnlijk niet primair verantwoordelijk is voor de inductie van symptomen, hebben wij ons geconcentreerd op de eigenschappen van het door TMV geinduceerde 116 kDa-eiwit. Na inoculatie van oudere, volgroeide bladeren kon TMV 96 uur later in de systemisch geinfecteerde topblaadjes worden waargenomen; het 116 kDa-eiwit tussen de 120 en 144 uur na inoculatie. Dit was tevens het moment waarop zich de eerste symptomen ontwikkelden. Op dit tijdstip was het 116 kDa-eiwit eveneens aanwezig in de oplosbare eiwitfractie en geassocieerd met membranen. Het 116 kDa-eiwit leek bij voorkeur geassocieerd met, aan de ene kant, de membraanfractie en, aan de andere kant, chromatine. Op grond van de hoeveelheid van het 116 kDa-eiwit in de diverse fracties en de uit metingen met het microscoop verkregen volumina van de diverse celorganellen kon berekend worden dat de concentratie in de kern ongeveer 8× hoger was dan in het cytoplasma. Voorts was, in tegenstelling tot bij TMV-manteleiwit, voor het verbreken van de associatie tussen het 116 kDa-eiwit en chromatine natriumchloride noodzakelijk. Dit suggereerde dat het eiwit relatief sterk geassocieerd was met chromatine en mogelijk net als de niet-histon-chromatine-eiwitten een rol zou kunnen vervullen bij de regulatie van de specifieke genexpressie. (Hoofdstuk 4).

Daar bekend is dat TMV codeert voor een eiwit met ongeveer gelijke molecuulmassa, werd nagegaan of het nieuwe 116 kDa-chromatine-eiwit uit mozaiekzieke tabaksbladeren wellicht identiek is aan het door TMV gecodeerde 126 kDa-eiwit. Daartoe werd in de reageerbuis nagebootst wat normaal in de cel gebeurt, namelijk

het omzetten van het virale RNA in eiwit. Dit eiwit kan zo met behulp van radioactieve aminozuren gemerkt worden. Dit *in vitro* gesynthetiseerde eiwit werd vervolgens in polyacrylamidegels vergeleken met het 116 kDa-eiwit afkomstig van het chromatine uit de met TMV geinfecteerde plant. Zowel de hele eiwitten als de fragmenten die na gedeeltelijke afbraak met het eiwitsplitsende enzym protease V8 werden verkregen, bleken op dezelfde plaatsen in polyacrylamide gels terecht te komen. Dit vormt een bewijs dat beide eiwitten inderdaad identiek zijn (Hoofdstuk 5).

In hoofdstuk 6 wordt een verbeterde methode voor de isolering van chromatine eiwitten beschreven. Hiervan werd gebruik gemaakt voor de karakterisering, door middel van twee-dimensionale polyacrylamide-gelelektroforese, van de chromatine-eiwitpatronen van tabakssoorten en -varieteiten met verschillende genetische samenstelling. Met name de toepassing van twee fenolextracties gevolgd door neerslaan van de geëxtraheerde eiwitten met zeer koude alcohol, verhoogden de opbrengsten aan eiwit aanzienlijk. Tweedimensionale gelelektroforese van de aldus verkregen eiwitmonsters resulteerde in scherpere en intensere eiwitvlekken op gel vergeleken met de voordien gebruikte eiwitisolatiemethode. Meer dan 90% van de eiwitten die aanwezig waren in de diverse tabakssoorten en -varieteiten leken identiek. Onder de 10% van de eiwitten waarin zij verschilden, kon(den) geen specifieke spot(s) gerelateerd worden met de aanwezigheid van het gen *N*, dat betrokken is bij de lokaliseringsreactie volgend op infectie met TMV.

In Hoofdstuk 7 tenslotte, wordt de mogelijke betrokkenheid van het 116 kDa eiwit bij de ontwikkeling van symptomen bediscussieerd. Op grond van waarnemingen dat blokkering van de aflezing van plantegenen met behulp van de remmer actinomycine D, in zich ontwikkelende bladeren soortgelijke symptomen induceert als TMV-infectie, wordt in dit hoofdstuk gespeculeerd over de mogelijkheid dat het 116 kDa-eiwit optreedt als repressor van die plantegenen die specifiek vroeg in de bladontwikkeling werkzaam zijn.

Voorts werd elders recentelijk vastgesteld dat tussen het TMV-gecodeerde 116 kDa-eiwit en eiwitten van ongeveer dezelfde grootte uit twee niet-verwante plantevirussen, het luzernemozaiekvirus en het 'brome mosaic virus', een overeenkomst van 20-30% in de volgorde van de aminozuren bestaat. Dit suggereert dat de interactie van andere plantevirussen met hun gastheer mogelijk op een soortgelijke wijze verloopt als TMV infectie van tabak.

Curriculum Vitae

Hendrik Jan van Telgen werd op 19 juni 1954 geboren te Velsen. Hij bezocht de Prof. Mr. S. Vissering Scholengemeenschap te IJmuiden waar hij in 1971 het einddiploma HBS-b behaalde. In datzelfde jaar werd met de studie Moleculaire Wetenschappen aan de Landbouwhogeschool begonnen. Het doctoraalexamen werd in juni 1979 afgelegd, met als hoofdvakken biochemie en genetica en als bijvak virologie. De praktijdtijd werd van april tot en met november 1977 doorgebracht bij de vakgroep Genetica van de Universiteit van Konstanz (Bondsrepubliek Duitsland). Na vervulling van de militaire dienst was hij van 1 januari 1981 tot en met 31 december 1983 als wetenschappelijk assistent werkzaam bij de vakgroep Plantenfysiologie van de Landbouwhogeschool, waar onder leiding van dr. ir. L. C. van Loon dit proefschrift bewerkt is. Gedurende deze drie jaar was hij gestationeerd bij de vakgroep Virologie.