Soluble Proteins as Genetic Markers in Studies of Resistance and Phylogeny in *Nicotiana*

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ABSTRACT

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New soluble proteins (b-proteins) appear in *Nicotiana* when resistance is induced either by a hypersensitive reaction to infection or by treatment with chemicals. Determination of their patterns in several species and cultivars has revealed both interspecific and intraspecific variations. The b-proteins of *Nicotiana sylvestris* (R_f's: $b_0 = 0.88$, $b_1 = 0.83$, $b_3 = 0.56$), *Nicotiana tomentosiformis* (R_f: $b_2 = 0.66$) and of 16 cultivars of *Nicotiana tabacum* (R_f's: $b_1 = 0.83$, $b_2 = 0.66$, $b_3 = 0.56$ and, in five cultivars, $b_{1'} = 0.79$) are charge isomers with a molecular weight of 15,700. Protein b_4 ($R_f = 0.53$), which is present in eight tobacco cultivars, has a molecular weight of 29,500. The determinants of these proteins are sexually transmitted and a monogenic inheritance has been demonstrated for one of them ($b_{1'}$). The hypothesis on the origin of present-day tobacco is strengthened by the observation that *N. tabacum* seems to have inherited b-proteins from both *N. sylvestris* (b_1 and b_3) and *N. tomentosiformis* (b_2). Resistance to TMV in *N. tabacum* comes from the introduction into this species of a resistance gene from *Nicotiana glutinosa*, the *N* gene. The b-protein found in this latter species, $b_{1''}$ ($R_f = 0.76$; mol wt = 13,800) differs from tobacco b-proteins. However, since the *N* gene is required for b-protein elicitation in tobacco after TMV infection, it is suggested that this resistance gene may play a regulatory role on the structural gene(s) coding for the b-proteins. The use of b-proteins as genetic markers for studies on resistance and for phylogenetic investigations is proposed.

Additional key words: *N. debneyi*.

The hypersensitive response of tobacco leaves to viral, fungal, or bacterial infections is accompanied by the appearance of new soluble host proteins, initially in the infected tissue, and later in the rest of the plant (1,14,16,28). Gianinazzi (12) proposed that these new proteins, called b-proteins, may play a role in the localization of the parasite as well as in the acquired resistance that develops in the plant following a hypersensitive reaction. Further evidence that a relationship exists between the presence of these new components and acquired resistance has been obtained by using synthetic inducers of resistance to tobacco mosaic virus (TMV), such as polyacrylic acid or aspirin. Following injection of these chemicals into the leaves, only tissues in which b-proteins have been elicited in significant amounts develop resistance to TMV (1,3,15,29).

A previous study of two cultivars of *Nicotiana* and their reciprocal hybrids has revealed that the number and the electrophoretic mobility of the b-proteins may differ according to the genetic constitution of the plant (13). Van Loon and Van Kammen (28) have also reported the presence of another protein, with an electrophoretic mobility differing from that of the tobacco b-proteins, in *N. glutinosa* following TMV inoculation. These two observations suggest that b-proteins could act as genetic markers and thus provide a new approach for studies of the resistance

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mechanisms in tobacco. In the present work, we have analyzed the b-protein patterns of several *Nicotiana* species, cultivars and hybrids to gain insight into the relationships between these proteins and the *N* gene that confers resistance to TMV. Mendelian segregation of determinants for one of the b-proteins is reported for the first time and the use of such studies for investigating phylogenetic relationships in the genus *Nicotiana* is proposed.

MATERIALS AND METHODS

Plants. The following plants were used: *Nicotiana tomentosiformis, N. sylvestris, N. glutinosa, N. debneyi*, and *N. tabacum* 'Amersfort,' 'Burley 21,' 'Burley 49,' 'Cridlo Corentino,' 'Izmir,' 'Judy's Pride Burley,' 'Kentucky Pulawy,' 'Maryland 10,' 'NC 95,' 'Paraguay P48,' 'PBD 6,' 'Samsun NN,' 'Samsun nn,' 'SC 58,' 'Xanthi-nc' (from P. Schiltz, Experimental Institute of Tobacco, Bergerac, France), and 'X 73' (from R W. Fulton, University of Wisconsin, Madison 53706). The amphidiploid *N. sylvestris* × *N. tomentosiformis* was also studied, as well as several intraspecific and interspecific hybrids between the plants cited above. Plants were grown in a greenhouse and used in the experiments when they were 10 to 13 wk old.

Viruses. Tobacco mosaic virus (TMV) common strain or aucuba (from E. Boudon, I.N.R.A., Dijon, France) and tobacco necrosis virus (from M. Conti, Fytovirology Laboratory, CNR, Turin, Italy) were used to elicit a hypersensitive reaction; TMV, when not otherwise specified, refers to the common strain. These viruses were inoculated by rubbing the surface of Carborundum-dusted leaves with the sap of infected leaves. Water-inoculated leaves served as controls.

Polyacrylic acid. Since *N. tomentosiformis* is sensitive to TMV (systemic infection) and repeated attempts to inoculate it mechanically with tobacco necrosis virus (TNV) always failed, a synthetic inducer of resistance, polyacrylic acid (PAA) (Egachemie-Steinheim/ Albuch, West Germany) was used to elicit b-proteins in this species. The petioles of detached leaves were placed either in a 0.01% PAA (mol wt = 2,000) solution or in water (controls) for 7 days.This concentration and molecular weight of PAA was chosen, in contrast to those employed in previous studies (3,15), because preliminary experiments carried out on different species and cultivars showed that the b-protein patterns obtained under these conditions do not differ from those obtained after a hypersensitive reaction.

Plant protein extraction. Tissue samples (1 g) from control, virus-inoculated, or PAA-treated leaves were harvested after 7 days and ground in 1 ml of phosphate-citrate buffer, pH 2.8, and 1% 2-mercaptoethanol, as described by Gianinazzi et al (17). After centrifugation at 10,000 *g* for 30 min, the supernatant fluids were collected and dialyzed overnight against 0.1 M Tris-HCl buffer, pH 8.0, containing 0.1% 2-mercaptoethanol.

Electrophoresis. After dialysis, extracts were electrophoresed in 10, 11, 12, 13, or 14% polyacrylamide gels, according to the method of Davis (9), with a running buffer of pH 8.3. Gels were stained overnight with 0.03% Coomassie blue R 250 in a methanol, acetic acid, water mixture (5:1:5, v/v) and destained in 10% acetic acid. Protein band patterns were traced using a Vitatron TLD-100 densitometer and the migration of the b-proteins (R_f) was determined relative to the distance traveled by plant phenols.

Molecular weight determinations. The proteins of the plant extracts were separated by electrophoresis in 10% polyacrylamide gels, as described above. Each b-protein was sliced from the gels in a 2-mm-thick disk and extracted from the gel by maceration in a Tris-phosphate buffer, pH 6.7, containing 1% sodium dodecyl sulfate (SDS) and 0.5% 2-mercaptoethanol. Polyacrylamide was eliminated by centrifugation and the supernatant fluids were electrophoresed in a 12% polyacrylamide vertical slab, pH 8.9, plus 0.1% SDS, with a stacking gel of 5% polyacrylamide, pH 6.7, plus 0.1% SDS. After staining with 0.1% Coomassie blue R 250, migration of proteins relative to that of bromophenol blue was recorded. Marker proteins used in the vertical slab gels were TMV coat protein (mol wt = 17,700, provided by R. Scalla, I.N.R.A., Dijon, France) and horse heart cytochrome c (mol wt = 12,400), human erythrocyte carbonic anhydrase (mol wt = 29,500), beef heart lactate dehydrogenase (mol wt = 36,000), and chicken ovalbumin (mol wt = 43,000), all obtained from Sigma (Sigma Chemical Co., St. Louis, MO 63178).

RESULTS

The variability of b-protein patterns in tobacco cultivars and *Nicotiana* spp. Results obtained are presented in Fig. 1 and Table 1. As previously shown, the hypersensitive reaction in *N. tabacum* 'Xanthi-nc' was followed by the appearance of four new soluble leaf proteins, with R_f's of 0.83, 0.66, 0.56, and 0.53 and called b_1, b_2, b_3, and b_4, respectively (16) (Fig. 1A and B). The same b-protein pattern was found in six other tobacco cultivars, and was confirmed by coelectrophoresis. Only proteins b_1, b_2, and b_3 were detected in four cultivars. In five other cultivars, one additional new protein was found (see for example the soluble protein pattern of Paraguay P48 tobacco, Fig. 1C and D) (Table 1). This protein had an intermediate R_f of 0.79 and was called $b_{1'}$. It cannot be concluded that the b_4 protein is not present in all cultivars in Table 1. Since it was found in low concentration, it may have been masked by other host proteins in the same position on the gels.

In addition to this intraspecific variability, b-protein patterns also varied interspecifically. In *N. tomentosiformis*, one new protein, with an R_f of 0.66, which corresponded to that of the b_2 protein, was present in the extracts from PAA-treated leaves (Fig. 1E and F). In *N. sylvestris*, proteins with R_f values similar to those of b_1 (0.83) and b_3 (0.56) were detected after TNV inoculation, as well as another more rapidly migrating protein (R_f = 0.88) termed b_0 (Fig. 1G and H). Coelectrophoreses of these extracts with that of *N. tabacum* 'Xanthi-nc' confirmed the homology between proteins b_1, b_2, and b_3 of these different species.

TMV-infected *N. glutinosa* (Fig. 1I and J) and TNV-infected *N. debneyi* (Fig. 1K and L) both contained only one additional protein not present in uninfected control plants. This protein differed from all the others and was called $b_{1''}$ because its R_f was intermediate between those of proteins $b_{1'}$ and b_2.

Inheritance of b-protein determinants. The b-protein patterns of a number of intraspecific and interspecific hybrids and the amphidiploid *N. sylvestris* × *N. tomentosiformis* were studied. All of these hybrids contained the sum of the b-proteins present in the parents, independently of the direction of the cross. This shows that the determinants of the b-proteins are sexually transmitted (Table 2).

Inheritance of the $b_{1'}$ protein was studied further in the descendants of the hybrid Burley 49 × Judy's Pride Burley and in relation to the *N* gene, which confers hypersensitivity to TMV. This gene is present in Burley 49 tobacco, but absent from Judy's Pride Burley. This hybrid was backcrossed with either Burley 49 or Judy's

TABLE 1. Soluble protein (b-protein) patterns of 16 *Nicotiana tabacum* cultivars, as revealed by analysis in 10% polyacrylamide gels

Cultivar	Necrosis-inducing viruses	b-Proteins[a]				
		b_1	$b_{1'}$	b_2	b_3	b_4
Xanthi-nc	TMV, TNV	+	−	+	+	+
Burley 21	TMV, TNV	+	−	+	+	+
Kentucky Pulawy	TNV	+	−	+	+	+
NC 95	TNV	+	−	+	+	+
Samsum NN	TMV, TNV	+	−	+	+	+
Samsun nn	TNV	+	−	+	+	+
X 73	TMV, TNV	+	−	+	+	+
Burley 49	TMV, TNV	+	−	+	+	−
Maryland 10	TNV	+	−	+	+	−
PBD 6	TMV, TNV	+	−	+	+	−
SC 58	TNV	+	−	+	+	−
Amersfort	TNV	+	+	+	+	−
Cridlo Corentino	TNV	+	+	+	+	−
Izmir	TNV	+	+	+	+	−
Judy's Pride Burley	TNV	+	+	+	+	−
Paraguay P48	TNV	+	+	+	+	+

[a]+ = Presence; − = absence.

Pride Burley, or self-fertilized. Plant offspring that were sensitive to TMV were inoculated with TNV to provoke a hypersensitive reaction and to determine their b-protein patterns.

Backcrosses with Judy's Pride Burley gave progeny of which 59% were hypersensitive to TMV and 41% were sensitive to TMV. Protein analysis of 20 randomly selected plants showed that they all contained the $b_{1'}$ protein. In contrast, progeny of the backcross with Burley 49 was 100% hypersensitive to TMV, but segregation of the $b_{1'}$ protein occurred; this protein was present in only 48% of the plants (Table 3).

Self-fertilization experiments gave the following results: 21% of the progeny were sensitive to TMV and 79% were hypersensitive,

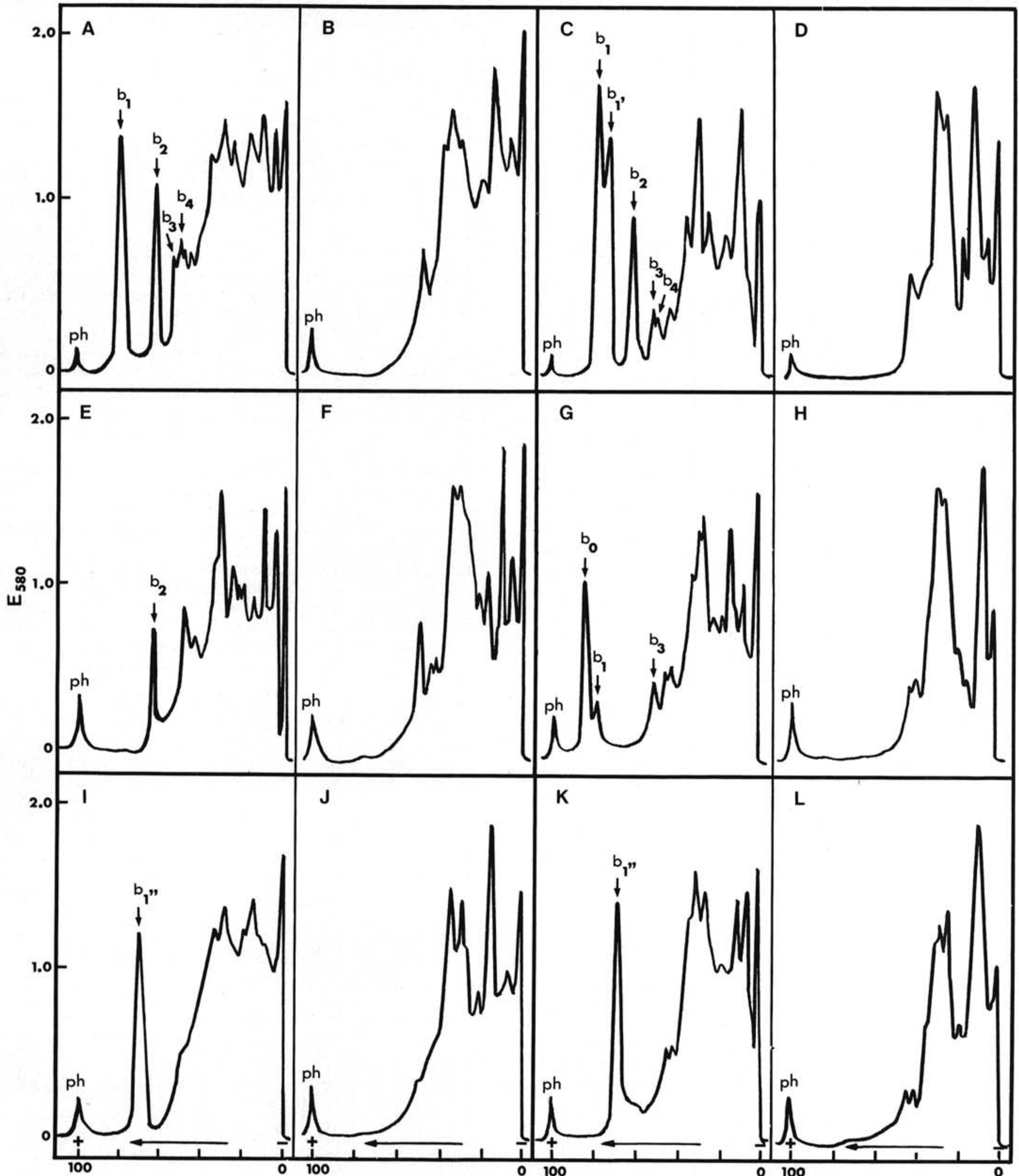

Fig. 1. Densitometer tracings of soluble leaf proteins separated in 10% polyacrylamide gels in extracts from 7-day infected leaves (**A, C, G, I, K**), 7-day PAA-treated leaves (**E**), and control leaves (**B, D, F, H, J, L**). *Nicotiana tabacum* 'Xanthi-nc' (**A, B**), *N. tabacum* 'Paraguay P48' (**C, D**), *N. tomentosiformis* (**E, F**), *N. sylvestris* (**G, H**), *N. glutinosa* (**I, J**), and *N. debneyi* (**K, L**). New proteins are indicated by the letter b; ph = plant phenols.

and the $b_{1'}$ protein was present in 76% of the plants (Table 3). Analysis by χ^2 test of these results showed that the information coding for the $b_{1'}$ protein follows a monogenic segregation, as does the *N* gene (20,21), but that these two characters are inherited independently.

Biochemical characterization of b-proteins. Molecular weights of b-proteins were determined using SDS polyacrylamide gel electrophoresis. Proteins b_0, b_1, b_3 from *N. sylvestris*, b_2 from *N. tomentosiformis* and b_1, $b_{1'}$, b_2, and b_3 from *N. tabacum* 'Judy's Pride Burley' and 'Burley 49' and from their reciprocal hybrids each gave a single band in SDS gels and all were located at the same position. This indicates that they are all composed of one or more monomers of the same size. The calculated molecular weights for these monomers ranged between 15,500 and 16,000, with a mean value of 15,700 (Fig. 2). This value is similar to those obtained for proteins b_1, b_2, and b_3 of Xanthi-nc (17) and Samsun NN (2) tobaccos.

A single band was also obtained in SDS gels for protein $b_{1''}$ of *N. glutinosa* and of *N. debneyi*. This had a higher relative migration and the calculated molecular weight was 13,800 (Fig. 2).

For further characterization of b-proteins, relative migration of proteins b_0, b_1, $b_{1'}$, b_2, and b_3 of *N. sylvestris*, *N. tomentosiformis*, *N. tabacum* 'Burley 49,' 'Judy's Pride Burley,' 'Samsun NN,' 'Samsun nn,' and 'Xanthi-nc' were determined in 10, 11, 12, 13, or 14% polyacrylamide gels without SDS. The logarithms of the R_f values obtained for each b-protein were plotted against polyacrylamide gel concentration and a linear relationship was obtained. There were no statistically significant differences among the slopes obtained for each of these b-proteins (Table 4). According to Hedrick and Smith's (19) theory, this, together with the fact that all these b-proteins comigrate in SDS gels, means that, in their native form, b_0, b_1, $b_{1'}$, b_2, and b_3 are charge isomers. From these results and those recently published by Antoniw et al (2), who have shown by ultracentrifugation that proteins b_1, b_2, and b_3 of *N. tabacum* 'Samsun NN' and 'Xanthi-nc' are composed of a single polypeptide, we can conclude that, in their native form, all these b-proteins of *N. sylvestris*, *N tomentosiformis*, *N. tabacum* 'Burley 49,' 'Judy's Pride Burley,' 'Samsun NN,' 'Samsun nn,' and 'Xanthi-nc' are monomers with different charges.

The slopes obtained for protein b_4 from Samsun NN, Samsun nn, and Xanthi-nc tobaccos were always significantly steeper than those for the other b-proteins. This confirms a previous report (17) that this protein has a higher molecular weight (29,500).

No statistical difference was found between the slopes obtained for protein $b_{1''}$ of *N. glutinosa* and that of *N. debneyi*; since these two proteins have the same molecular weight, they must be either identical or closely similar. Because the slopes obtained for these $b_{1''}$ proteins are very close to those for the other b-proteins (except for b_4), the former are probably also monomers in their native forms.

DISCUSSION

The number and electrophoretic mobility of soluble proteins appearing in the genus *Nicotiana* after a hypersensitive reaction differ both intraspecifically and interspecifically. It is, therefore, possible to use them as specific genetic markers, with the reservation that the same b-protein pattern is sometimes found in different species, as is the case for *N. glutinosa* and *N. debneyi*. The use of b-proteins as varietal markers seems more difficult, since up to now, the only certain variation found within the different tobacco cultivars is the presence or the absence of the $b_{1'}$ protein.

The hypothesis that present-day tobacco has originated from an hybrid between ancestral forms of *N. sylvestris* and *N.*

TABLE 2. Soluble protein (b-protein) patterns of five intraspecific and six interspecific hybrids between *Nicotiana*, as revealed in 10% polyacrylamide gels

Hybrid	Necrosis-inducing viruses[a]	b-Proteins[b]						
		b_0	b_1	$b_{1'}$	$b_{1''}$	b_2	b_3	b_4
Intraspecific tobacco hybrids:								
Burley 49 × Judy's Pride Burley	TMV, TNV	−	+	+	−	+	+	−
Samsun NN × Judy's Pride Burley	TMV, TNV	−	+	+	−	+	+	+
Samsun nn × Burley 49	TMV, TNV	−	+	−	−	+	+	+
Samsun nn × Judy's Pride Burley	TNV	−	+	+	−	+	+	+
Xanthi-nc × Judy's Pride Burley	TMV, TNV	−	+	+	−	+	+	+
Interspecific hybrids:								
N. debneyi × *N. glutinosa*	TMV, TNV	−	−	−	+	−	−	−
N. tabacum 'Judy's Pride Burley' × *N. glutinosa*	TMV, TNV	−	+	+	+	+	+	−
N. tabacum 'Judy's Pride Burley' × *N. sylvestris*	TMVa, TNV	+	+	+	−	+	+	−
N. tabacum 'Samsum NN' × *N. glutinosa*	TMV, TNV	−	+	−	+	+	+	+
N. tabacum 'Samsun NN' × *N. sylvestris*	TMV, TNV	+	+	−	−	+	+	+
N. sylvestris × *N. tomentosiformis*	TMVa, TNV	+	+	−	−	+	+	−
Amphidiploid:								
N. sylvestris × *N. tomentosiformis*	TMVa, TNV	+	+	−	−	+	+	−

[a]TMVa = TMV aucuba.
[b]+ = Presence; − = absence.

TABLE 3. Inheritance of the *N* gene and the b_1 protein in the progeny of the hybrid Burley 49 × Judy's Pride Burley

Cross	Reaction to TMV			Occurrence of the $b_{1'}$ protein		
	Number of plants tested	Observed ratio of hypersensitive: sensitive plants	Expected ratio[a] of hypersensitive: sensitive plants	Number of plants tested	Observed ratio of plants with: plants without protein $b_{1'}$	Expected ratio[a] of plants with: plants without protein $b_{1'}$
(Burley 49 × Judy's Pride Burley) × Judy's Pride Burley	96	57:39	48:48	20	20:0	20:0
(Burley 49 × Judy's Pride Burley) × Burley 49	100	100:0	100:0	50	24:26	25:25
(Burley 49 × Judy's Pride Burley) self-fertilized	119	94:25	89:30	50	38:12	37:13

[a]Expected ratio according to monogenic inheritance.

TABLE 4. Estimated slopes[a] for the lines obtained for each b-protein of nine *Nicotiana* species and cultivars, when logarithms of relative migrations in gels are plotted against polyacrylamide concentrations

Plant	Proteins						
	b_0	b_1	$b_{1'}$	$b_{1''}$	b_2	b_3	b_4
N. sylvestris	−3.88	−3.93	...	...	...	−4.10	...
N. tomentosiformis	...	...	...	...	−4.08	...	...
N. tabacum 'Burley 49'	...	−4.15	...	...	−4.04	−4.43	...
N. tabacum 'Judy's Pride Burley'	...	−4.10	−4.02	...	−4.09	−4.41	...
N. tabacum 'Samsun NN'	...	−4.17	...	...	−4.17	−4.41	−5.39*
N. tabacum 'Samsun nn'	...	−3.92	...	...	−4.06	−4.30	−5.25*
N. tabacum 'Xanthi-nc'	...	−4.06	...	...	−4.10	−4.37	−5.36*
N. glutinosa	...	...	...	−3.95	...	...	...
N. debneyi	...	...	...	−3.85	...	...	...

[a] Analysis of variance was used to compare, first, within a given species or cultivar, the slopes obtained for the different b-proteins (rows) and, second, the slopes obtained for a given b-protein in the different species or cultivars (columns). * = Value statistically different ($P<0.05$) from those in the same row.

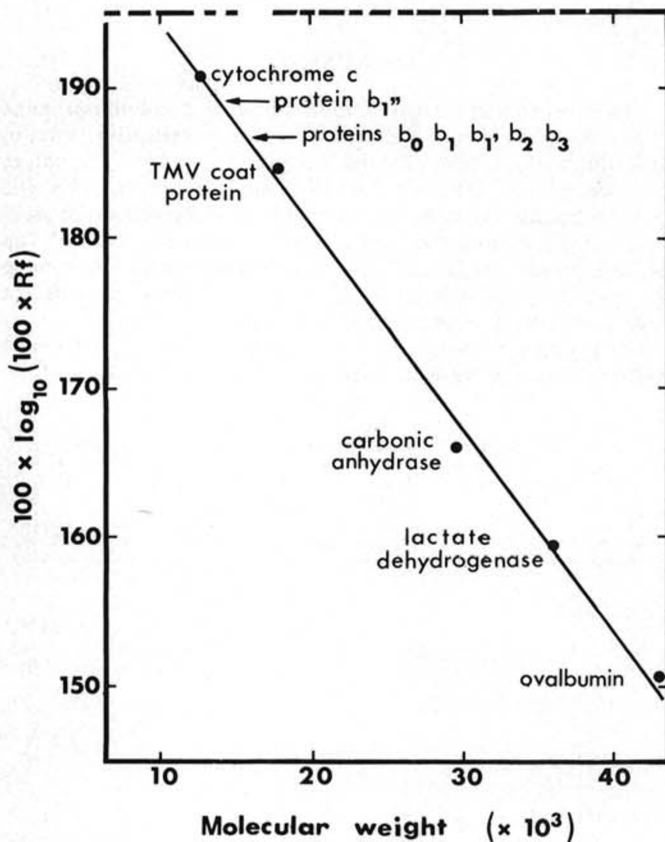

Fig. 2. Relative migration of markers of known molecular weights and b-proteins of *Nicotiana sylvestris* (b_0, b_1, and b_3), *N. tomentosiformis* (b_2), *N. tabacum* 'Burley 49' (b_1, b_2, and b_3) and 'Judy's Pride Burley' (b_1, $b_{1'}$, b_2, and b_3), *N. glutinosa* ($b_{1''}$) and *N. debneyi* ($b_{1''}$) in 12% polyacrylamide + 0.1% SDS gels.

tomentosiformis, whose chromosome content has been doubled to restore fertility (18), is already supported by many works: genetic (11) and cytogenetic (6,7,22) investigations, studies of isoenzyme patterns (26,27) and of nuclear DNA and heterochromatin (24), analysis of fraction 1 protein (23), reaction to tentoxin (5), and attempts to reconstruct self-fertile amphiploids from present-day *N. sylvestris* and *N. tomentosiformis* (4). Our results concerning the b-proteins seem to strengthen this hypothesis. Both hybrid and amphidiploid *N. sylvestris* × *N. tomentosiformis* contain the sum of the b-proteins from their two progenitors, b_0, b_1, b_2, and b_3, and all the *N. tabacum* cultivars tested so far contain the b_1, b_2, and b_3 proteins. Other *Nicotiana* species (*N. langsdorffii*, *N. glauca*, *N. sanderae* [*unpublished*], *N. glutinosa*, and *N. debneyi*) do not possess them. Thus, according to the hypothesis presented above, proteins b_1 and b_3 of present-day tobacco would have originated from *N. sylvestris* and b_2 from *N. tomentosiformis*. Molecular weight determinations of all these proteins and studies of their electrophoretic mobilities in gels with different concentrations of polyacrylamide confirm the homology that exists between b-proteins of *N. tabacum* and those of its hypothetical parents.

Proteins b_0, b_1, $b_{1'}$, b_2, and b_3 are charge isomers; their determinants (including that of protein b_4) are sexually transmitted and a monogenic inheritance has been demonstrated for one of them ($b_{1'}$). In *N. tabacum*, these determinants seem to be of different origin (*N. sylvestris* and *N. tomentosiformis*). These observations suggest that different genes may code for the different b-proteins. In this case, the absence of the b_0 protein from *N. sylvestris* in all the *N. tabacum* studied could be explained by loss of the corresponding gene during evolution of this latter species, or by recent appearance of the gene in present-day *N. sylvestris*. Investigations are being carried out in an attempt to clarify this point.

Much evidence indicates that b-proteins may be involved in an active defence mechanism against viruses in *Nicotiana* species, especially since it has not yet been possible to dissociate their presence from resistance in a tissue (1,3,29). This and the present observations indicate that genetic studies could be useful to extend understanding of active mechanisms of resistance in plants. The *N* gene, which governs the hypersensitive reaction to TMV, has been introduced into tobacco by replacement of the H chromosome with the Hg chromosome of *N. glutinosa* (10,21). This gene is necessary for b-protein appearance after TMV infection; tobacco b-proteins, however, are completely different from that of *N. glutinosa*. That means that the determinant for the b-protein of *N. glutinosa* has not been transferred with the Hg chromosome carrying the *N* gene and that the introduced *N* gene allows expression of the preexisting structural gene(s) coding for the b-proteins. That the b-proteins and the *N* gene are not linked is further confirmed by the independent segregation of the resistance gene from at least one of the b-proteins, $b_{1'}$, in the progeny of the hybrid Burley 49 × Judy's Pride Burley. It can therefore be hypothesized that the *N* gene has a regulatory role on the expression of the structural gene(s) coding for the b-proteins, in response to TMV infection. As genetic markers, b-proteins could be useful for further studies on both the role they may play in resistance and the regulation of their appearance.

Reports on the existence of similar resistance-related proteins in other plant families (8,12,25) further enhances the interest of b-proteins for studies of resistance and phylogeny.

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