ON THE NATURE OF THE POLYMORPHISM OF THE SMALL SUBUNIT OF RIBULOSE-1,5-DIPHOSPHATE CARBOXYLASE IN THE AMPHIDIPLOID NICOTIANA TABACUM

by

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The N-terminal amino acid sequence of the small subunit of ribulose-1,5-disphosphate carboxylase from the amphidiploid *Nicotiana tabacum* contains two polymorphisms. From examination of the equivalent sequences in the putative parent species *Nicotiana sylvestris* and *tomentosiformis* it is concluded that the amphidiploid *Nicotiana tabacum* has inherited two alleles for the small subunit of ribulose diphosphate carboxylase, one from each parent species. The alleles continue to be retained and expressed. The relevance of these findings is discussed in relation to the successful adaption of the amphidiploid *Nicotiana tabacum* to a wide range of environments.

1. INTRODUCTION

Among higher plants auto- and allopolyploids are highly successful as wild or cultivated species. According to F. VON WETTSTEIN (23) a selective advantage of polyploids lies in the possibiiity to harbour at a gene locus two or more different alleles and thereby to provide for a greater versatility of gene actions or – in analogy with duplicated genes in diploids – for diversification by mutation. As pointed out by FINCHAM (7) hetero-oligomeric enzyme molecules resulting from the presence of two different alleles at a locus may have selective advantages. Such heterozygosity can in a self-fertilising plant species only by maintained permanently at the tetraploid level.

There are by now many examples detected by gel electrophoresis that polyploid species express multiple forms of enzymes, and amphidiploids the enzyme forms of both parents as well as novel hybrid enzymes (cf. 10). *Nicotiana tabacum* (n = 24) is an amphidiploid species which has arisen by interspecific hybridization and chromosome doubling in the manner first proposed by WINGE (24). Analysis of isoenzyme patterns (21) have supported the conclusion reached by CLAUSEN (6) and KOSTOFF (18) that *Nicotiana sylvestris* (n = 12) and *Nicotiana tomentosiformis* (n = 12) are the diploid parent species of *Nicotiana tabacum.*

Comparative polypeptide analysis of ribulose-1, 5-diphosphate carboxylase (Fraction I protein, E.C. 4.1.1.39) by isoelectric focusing has been carried out by GRAY. KUNG, WILDMAN and SHEEN (12) for the large and small subunit of *Nicotiana tabacum, sylvestris, tomentosiformis* and *otophora* as well as newly produced amphidiploids of *Nicotiana sylvestris* x *tomentosiformis* and *Nicotiana sylvestris x otophora.* The large subunit of *Nicotiana tabacum* revealed three polypeptide bands with the same isoelectric points as the three bands from *Nicotiana sylvestris,* whereas the polypeptide pattern obtained from the large subunits of *Nicotiana tomentosiformis* or *otophora* were different. Since the genetic code for the large subunit is maternally inherited (5) it was concluded that *Nicotiana sylvestris* is the female parent of *Nicotiana tabacum.*

The small subunit of *Nicotiana sylvestris, tomentosiformis* and *otophora* give single bands with the isoelectric focusing procedure, whereas *Nicotiana tabacum* produces two bands of which one corresponds in its isoelectric point to that of *Nicotiana sylvestris* or *otophora* and the other to that of *Nicotania tomentosiformis* (12). Since the genetic code for the small subunit shows biparental inheritance in a Mendelian manner (17) the results of GRAY *et al.* indicate that *Nicotiana tabacum* has retained the allelle of *Nicotiana sylvestris* as well as the allele of *Nicotiana tomentosiformis* at the gene locus coding for the small subunit of ribulose diphosphate carboxylase.

In this paper we report on the N-terminal amino acid sequence of the small subunit of the enzyme from *Nicotiana sylvestris* and *tomentosiformis.* It is compared with the sequence previously established for *Nicotiana tabacum* by GIBBONS, STRØBÆK, HASLETT and BOULTER (9) in order to investigate if the polymorphism of the small subunit of ribulose diphosphate carboxylase in *Nicotiana tabacum* can be explained by the retention of the two alleles originating from the *sylvestris* and *tomentosiformis* genomes.

2. MATERIALS AND METHODS

2.1. Plant material

Plants of *Nicotiana sylvestris* and *tomentosiformis* were grown from seeds which were derived from self-pollinated flowers, under greenhouse conditions as described by KAWASHIMA and WILDMAN (17). Leaves were harvested 2-3 months after germination of the seeds.

2.2. Purification of ribulose-1,5-diphosphate carboxylase and separation of subunits

The purification and analysis of the small subunit of ribulose-1,5-diphosphate carboxylase from self-pollinated *Nicotiana tabacum* cv. Turkish Samsun was described earlier (9).

About 100 mg three times crystallized native protein from *Nicotiana sylvestris* or *tomentosiformis* obtained by the direct crystallization method of CHAN *et al.* (4), with modification in the case of *tomentosiformis* (12), were dissolved in 25 ml 0.05 M Tris-HCl pH 8.5 buffer containing 0.5% sodium dodecyl sulphate and 0.14 M β-mercaptoethanol, flushed with nitrogen and incubated at room temperature for 30 minutes. The subunits were then separated by sodium dodecyl sulphate column chromatography under conditions described earlier (22). The sodium dodecyl sulphate was removed by precipiting the protein by acetone and subsequent dialysis against 0.05 M NH_3 (20).

2.3. Amino acid sequence determination

Edman degradation of 5 mg small subunit of *Nicotiana tabacum* was carried out with a Beckman 890C automatic sequencer using the fast protein program (No. 072172C). *Nicotiana sylvestris* and *tomentosiformis* were sequenced using a modification of program No. 122974 (2). This program was modified by the addition of a second cleavage reaction since the protein appeared to be less soluble in the heptafluorobu-

tyric acid. Resultant anilinothiazolinones were converted to the corresponding phenylthiohydantoins and identified by initial thin layer chromatography using the solvent systems of JEPPSSON and SJÖQUIST (15) followed by the appropriate separation on either gas liquid chromatography or polyamide-layer chromatography of their converted free amino acid after dansylation (13).

2.4. Determination of C-terminal sequence with carboxypeptidase A

Reduction and carboxymethylation was carried out as described by HIRS (14). The small subunit from *Nicotiana tabacum* was carboxymethylated, the small subunits from *sylvestris* and *tomentosiformis* were unmodified. The enzyme solution was prepared from a suspension of crystals of diisopropylfluorophosphate treated carboxypeptidase A (Sigma) according to the method of HARRIS (1,8).

The reaction solution contained 200 nmoles of protein sample in 600 µl N-ethylmorpholine acetate-buffer pH 8.5, 200 µl 1 nM norleucine (as internal standard) and 160 µl (160 µg) carboxypeptidase A. The reaction was carried out at 37° and aliquots (90 µl) were removed after 0, ¼, ½, 1, 2, 4, 7, 10 and 21 hours. The reaction was stopped by adding 30 µl 1 N HCl and the samples dried. After resuspension in 50 µl water, 5 µl 30% 5-sulphosalicylic acid were added to precipitate the protein, which was then removed by centrifugation. The supernatant was collected and neutralized with NaOH. 20 µl samples of the neutralized supernatant were applied directly onto the Durrum D-500 amino acid analyzer.

2.5. Amino acid analysis

Duplicate samples containing 30 µg protein and 20 nmoles norleucine were hydrolysed in 50 µl 6 N redestilled HCl containing 0.05% phenol for 24, 48 or 72 hours at 110°C in evacuated ampoules and analyzed on a Durrum D-500 automatic amino acid analyzer. Values for Thr and Ser were those extrapolated to zero time. Values for Val and Ile are those for 72 hours hydrolysis. Trp content was calculated after ultraviolet spectral analysis of 0.2 mg/ml protein samples in 0.1 N NaOH using the method of BENCZE and SCHMID (3).

Values of the remaining amino acids are expressed as an average for the total of 6 values for each protein.

Table I

Amino acid composition of the small subunits of ribulose-1,5-diphosphate carboxylase expressed as moles/100 moles

	Nicotiana sylvestris	Nicotiana tomentosiformis	Nicotiana tabacum
Asx	7.83	6.98	7.54
Thr	4.18	4.19	4.49
Ser	3.74	4.11	4.50
Glx	15.86	15.49	14.98
Pro	6.75	6.32	6.29
Gly	5.87	7.44	7.03
Ala	4.79	5.18	5.85
Cys	2.19	1.55	1.76
Val	6.66	5.94	6.41
Met	1.47	1.46	1.58
Ile	4.55	4.63	4.72
Leu	8.22	8.30	8.15
Tyr	7.88	8.84	7.57
Phe	4.19	4.19	3.92
His	0.79	0.15	0.44
Lys	6.91	6.58	6.82
Arg	3.58	4.18	3.86
Trp	4.53	4.49	4.09

3. RESULTS AND DISCUSSION

The amino acid composition of the small subunits from *Nicotiana tabacum, sylvestris* and *tomentosiformis* are shown in Tables I and II. In Table I the observed amino acid compositions are expressed in moles per 100 moles amino acid residues, the average standard error being 3% of the mean values. In Table II the calculated numbers of amino acid residues per 12,000 daltons small subunit polypeptide are given.

With regard to the majority of amino acids, the amino acid composition of the small subunit from *Nicotiana tabacum* is compatible with the expectation that it comprises an equal mixture of *sylvestris* and *tomentosiformis* polypeptides. The exceptions are Ala and Tyr. The subunit from *Nicotiana sylvestris* contains more Asx, Val and His than *tomentosiformis*. On the other hand *Nicotiana tomentosiformis* has a higher content of Gly and Tyr. Assuming a molecular weight of 12,000 (Table II) for the small subunit (16) these differences amount to one molecule of Asx, Val and His as well as to a difference of two molecules of Gly and one molecule of Tyr.

Table II

Amino acid composition of the small subunits of ribulose-1,5-diphosphate carboxylase expressed as residues/12,000 daltons polyptide.

	Nicotiana sylvestris	Nicotiana tomentosiformis	Nicotiana tabacum
Asx	8	7	7½
Thr	4	4	4-5
Ser	4	4	4-5
Glx	16	16	15-16
Pro	6-7	6-7	6-7
Gly	6	7-8	7
Ala	5	5	6
Cys	2	2	2
Val	7	6	6½
Met	2	2	2
Ile	5	5	5
Leu	8	8	8
Tyr	8	9	8
Phe	4	4	4
His	1	0	½
Lys	7	7	7
Arg	4	4	4
Trp	4-5	4-5	4

The N-terminal 21 residues of *Nicotiana tabacum, sylvestris* and *tomentosiformis* (Figure 1) are identical apart from positions 7 and 8. At position 7 *Nicotiana tabacum* is polymorphic for Ile and Tyr, whereas Ile alone is found in *sylvestris* and Tyr alone in *tomentosiformis*. At position 8 *Nicotiana tabacum* is polymorphic for Asn and Gly whereas Asn alone is found in *sylvestris* and Gly alone in *tomentosiformis*.

	5	10	15	20
N. sylvestris	Met Gln Val Trp Pro Pro Ile Asn	Lys Lys Lys Tyr Glu Thr Leu Ser	Tyr Leu Pro Asp Leu...	
N. tomentosiformis	Met Gln Val Trp Pro Pro Tyr Gly	Lys Lys Lys Tyr Glu Thr Leu Ser	Tyr Leu Pro Asp Leu...	
N. tabacum	Met Gln Val Trp Pro Pro Ile/Tyr Asn/Gly	Lys Lys Lys Tyr Glu Thr Leu Ser	Tyr Leu Pro Asp Leu...	

Figure 1. The N-terminal amino acid sequence of the small subunits of ribulose-1,5-diphosphate carboxylase from *Nicotiana sylvestris, tomentosiformis* and *tabacum*.

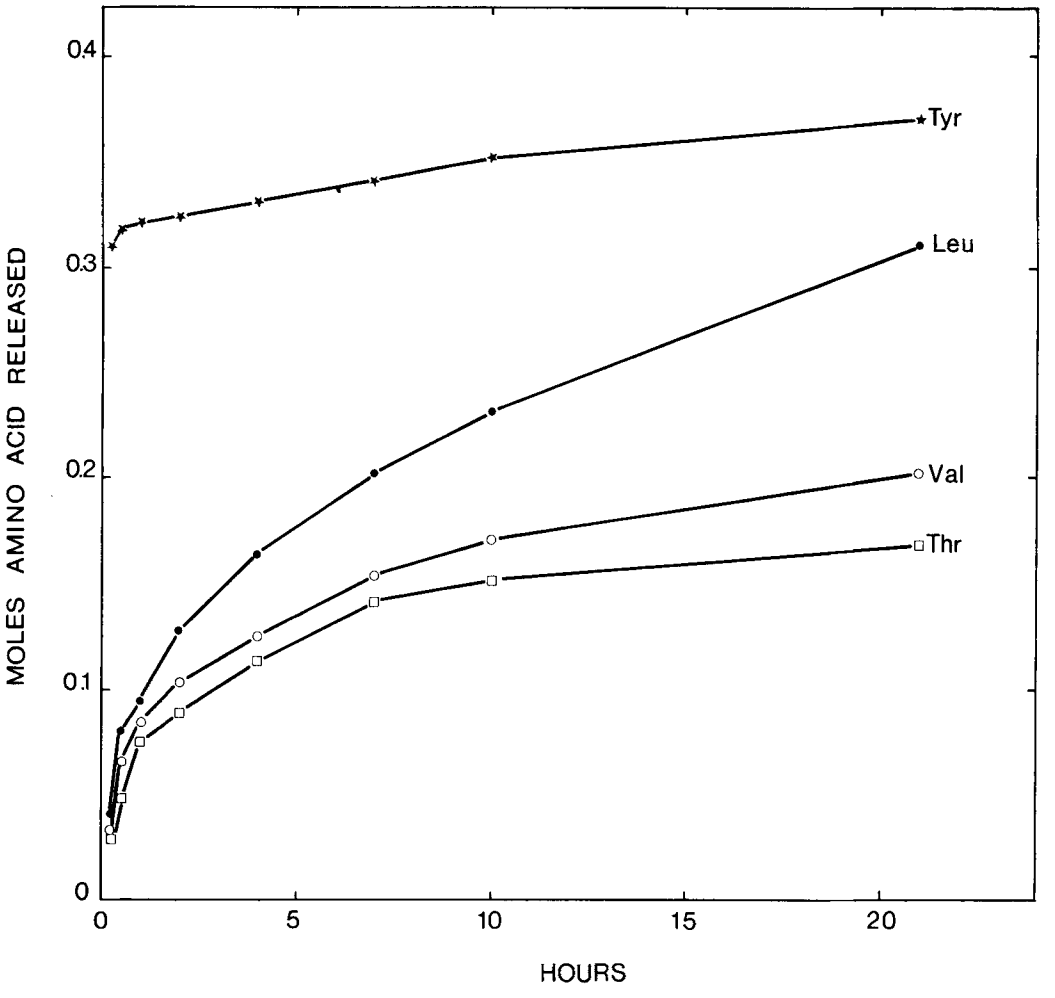

Figure 2. Course of digestion by carboxypeptidase A of carboxymethylated small subunit of ribulose-1,5-diphosphate carboxylase from *Nicotiana tabacum* cv. Turkish Samsun. The amount of released amino acid is expressed as moles per mole 12,000 daltons polypeptide.

The result of the digestion of the small subunit of ribulose disphosphate carboxylase from *Nicotiana tabacum* by carboxypeptidase A is shown in Figure 2. Comparable results were obtained for *Nicotiana sylvestris* and *tomentosiformis* indicating identical C-terminal sequences for the small subunit from all three species, namely: - Thr Val Leu Tyr-COOH.

The polymorphism of *Nicotiana tabacum* at positions 7 and 8 can be the result of a mixture of two polypeptides, one containing the sequence Ile Asn from *sylvestris* and the other Tyr Gly from *tomentosiformis* (cf. Figure 3). The differences in the isoelectric behavior of the two polypeptides resolvable in small subunit preparations of *Nicotiana tabacum* (12), (Figure 3) can not by explained by this sequence difference. However, *sylvestris* contains one histidine residue per polypeptide, whilst *tomentosiformis* lacks this basic amino acid (Table II), this being sufficient to account for the observed difference in isoelectric points of the small subunits. GRAY (11) has separated the two small subunit polypeptides of ribulose diphosphate carboxylase from *Nicotiana tabacum* by isoelectric focusing and examined their peptide maps following tryptic digestion. He found a unique basic peptide in the map of the *sylvestris* like polypeptide and a unique neutral peptide for the *tomentosiformis* like polypeptide.

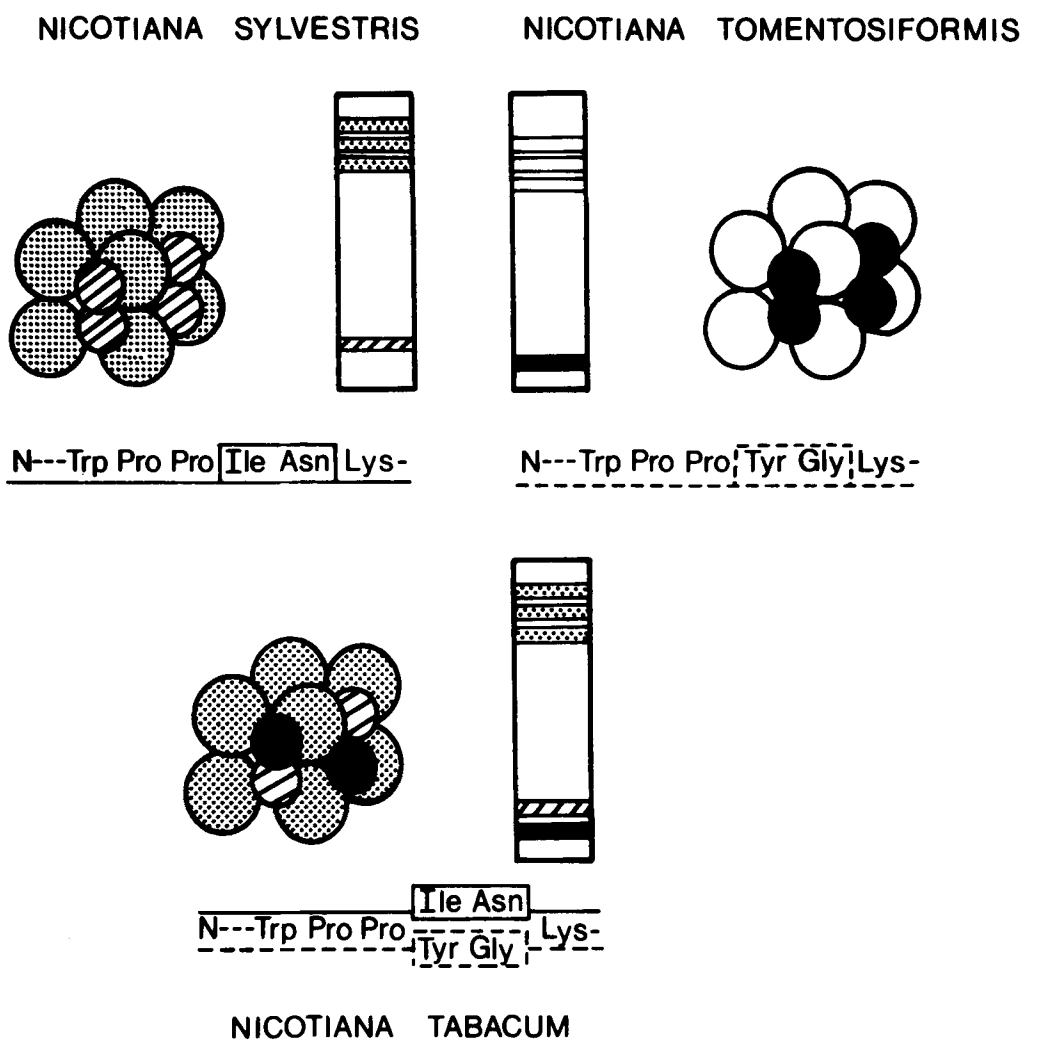

Figure 3. The nature of the polymorphism of ribulose-1.5-diphosphate carboxylase in *Nicotiana tabacum*. The porposed subunit structures of *Nicotiana sylvestris, tomentosiformis* and *tabacum* are presented in terms of 8 large + 8 small subunits configuration. The separations achieved by isoelectric focusing (12) are schematically shown in the columns beside the molecular models. The upper three bands originate from the large subunit, the lower from the small subunit. The sequences of the nine N-terminal amino acids are included to depict the sequence polymorphism described in the text.

Returning to the amino acid comparisions of the small subunits reported in Table II, the polymorphism in positions 7 and 8 can explain the species differences for Asx and Tyr. The presence of Ile in position 7 of the sequence of *Nicotiana sylvestris* is not expressed as an extra Ile in the total amino acid composition. *Nicotiana sylvestris* and *tomentosiformis* appear to differ by two Gly residues, one of which is due to the presence of Gly in position 8 of *tomentosiformis*. Thus at least two additional residues have to be polymorphic in the undetermined portion of the sequence of the small subunit from *Nicotiana tabacum*. The unknown part of the sequence of *Nicotiana sylvestris* is expected to contain a Val and His residue absent in *tomentosiformis*. Conversely, that of *Nicotiana tomentosiformis* should contain a Gly and Ile residue not present in *sylvestris*. By amino acid analysis the content of Ala was higher and the content of Tyr lower in *Nicotiana tabacum* than half of the combined values of these amino

acids in *sylvestris* and *tomentosiformis*. Further sequencing will reveal the basis for these differences.

In the initial report (9) on the sequence of *Nicotiana tabacum* small subunit, Gly was not included at position 8 since it occured only in small amounts together with Asn. At the time the significance of the presence of Gly was in doubt. Subsequent determinations confirmed the presence of low yields of Gly together with the high yields of Asn at this position. Gly is known to give lower yield due to incomplete conversion and to be easily obscured by the background of other derivatives (13).

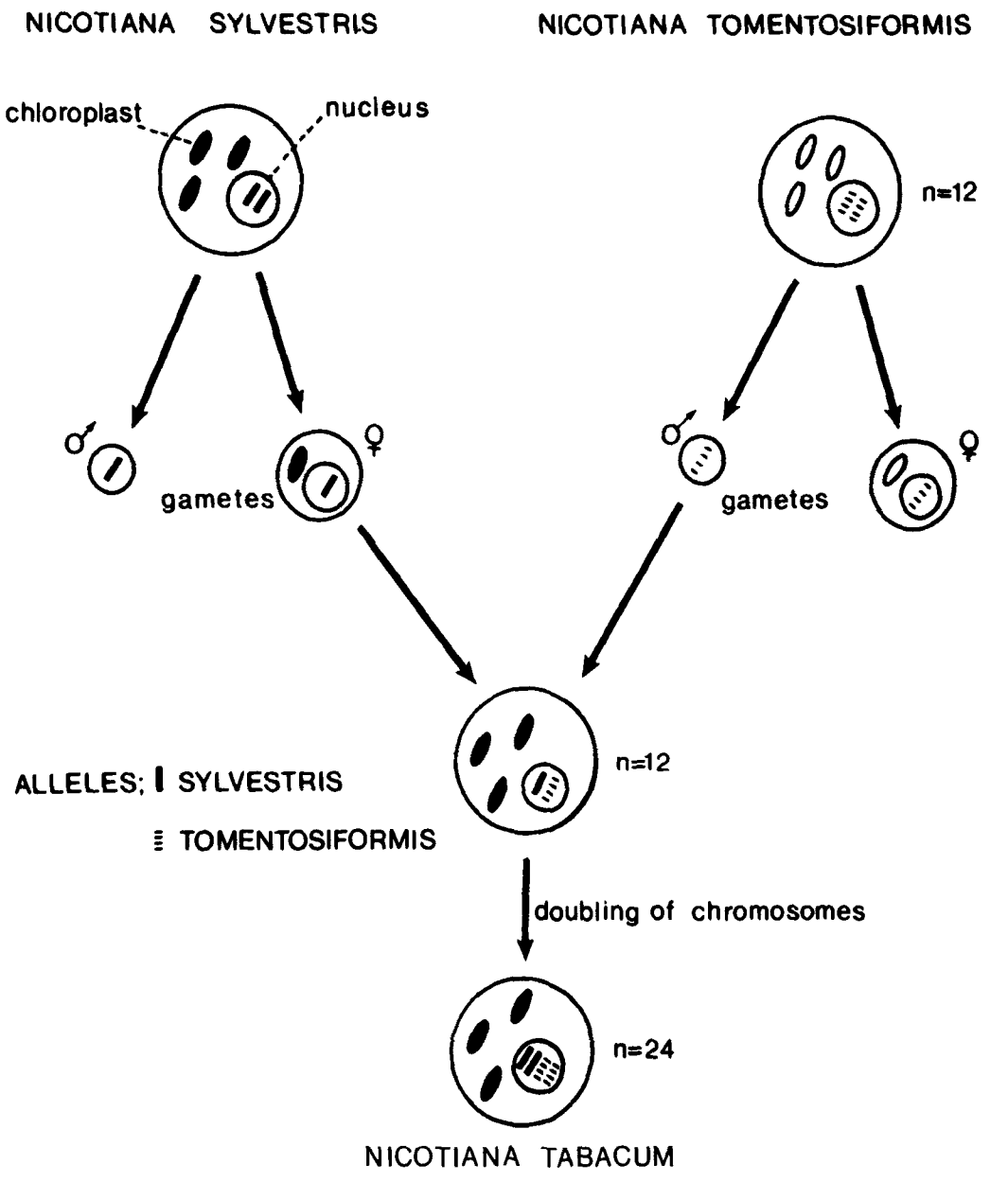

Figure 4. Model for the origin of the amphidiploid *Nicotiana tabacum* from *Nicotiana sylvestris* x *tomentosiformis*. The diagram shows that characters (large subunit of ribulose-1,5-diphosphate carboxylase (5)) determined outside the nucleus can only be inherited maternally, whereas the amphidiploid contains the total nuclear complement (including small subunit of ribulose-1,5-diphosphate carboxylase (17)) of both parents in allelic forms.

The primary structures of the N-terminal portion of the two types of small subunits of ribulose diphosphate carboxylase appear as the sum of the subunit sequences from *Nicotiana sylvestris* and *tomentosiformis*. Summation in *tabacum* of the amino acid substitutions present between *sylvestris* and *tomentosiformis* in two adjacent positions lend special credence to the theory on the origin of *Nicotiana tabacum* and argues strongly for the presence in *tabacum* of one pair of alleles for the small subunit from *sylvestris* and one pair of alleles from *tomentosiformis*, as shown in Figure 4.

The time of origin of *Nicotiana tabacum* is not known but the plant appears to have been cultivated for several hundred generations. During this time the genetic code for the small subunit of ribulose diphosphate carboxylase seems to have been preserved unaltered. From the evolutionary aspect the advent of hetero-oligomers caused by the combination of the two genes from the parent species does not seem to have been harmful to the new species. It will be interesting to analyse how general this phenomenon is, for instance by following the hybrid alcohol dehydrogenase in the amphidiploid species *Tragopogon mirus* and *miscellus* which have arisen during the last 60 years from *Tragopogon dubius* x *porrifolius* and *Tragopogon dubius* x *pratensis* and are successfully spreading in Washington and Idaho (10, 19).

The cultivation of *Nicotiana tabacum* has been successful in a variety of different climates and soil types. One is inclined to speculate, whether or not the availability to the chloroplast of 2 different homomeric forms and 9 heteromeric forms of ribulose diphosphate carboxylase confers a better adaptability to the plant. This problem may be attacked by seeking for physiological differences exhibited by these different molecular forms of the enzyme.

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