Molecular diversity in genus *Nicotiana* as revealed by randomly amplified polymorphic DNA

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Received 7 November 2007; revised 16 April 2008; accepted 25 June 2008

The genus *Nicotiana* consists of 64 recognized species of which, only 2 species, *N. tabacum* and *N. rustica* are cultivated extensively. Wild *Nicotiana* species are storehouses of genes for several diseases and pests, in addition to genes for several important phytochemicals and quality traits which are not present in cultivated varieties. Randomly amplified polymorphic DNA (RAPD) analysis was used to determine the degree of genetic variation in the genus *Nicotiana* and to develop species specific markers. 22 species and 2 interspecific hybrids were analyzed by using 18 decamer primers. Greater amount of genetic polymorphism exists among the wild species of genus *Nicotiana* (99.5%) as evidenced by the high degree of polymorphism in RAPD profiles. The pairwise similarity measures in the species of subgenus *Rustica* was 0.252 whereas in the subgenus *Tabacum* it was 0.189 suggesting that there was significant diversity amongst the species of these subgenera. In the species of subgenus *Petunioides*, the range of pairwise similarity measures was 0.128 to 0.941. The clustering pattern coincided with the traditional classification of *Nicotiana* species. All the primers generated specific bands in the various species. 36 species specific markers identified in the present study would be utilized in interspecific breeding programmes.

Keywords: *Nicotiana*, genetic diversity, RAPD

**Introduction**

The genus *Nicotiana* is one of the five major genera of the family *Solanaceae*. Of the total 64 species in the genus, 44 are indigenous to North or South America and 20 are native to Australia. The genus is divided into three sub-genera viz., *Rustica*, *Tabacum* and *Petunioides* mainly based on the morphology, crossability relationships, chromosomal number and behaviour in interspecific hybrids. Although, 6 chromosome paired species in *Nicotiana* are known, the predominance of 12 chromosome paired species indicates that 6 was the basic chromosome number for *Nicotiana* and both 12 paired, and 24 paired species are derived numbers. It is assumed that with a higher survival value, the allopoloids had eliminated the older 6-paired types. The 9-and 10-paired species are considered to have been evolved as a result of hybridization between the ancestral 6-paired and the present day 12-paired members. The 24-paired species including *N. tabacum* and *N. rustica* are modern descendents of the 12-paired progenitors entered into amphidiploid origin. *N. tabacum* and *N. rustica*, which are natural amphidiploids (2n=48) are grown commercially throughout the world. Although tropical in origin, tobacco can now be found growing from about 60°N to 45°S. Because of its economic importance and value as a biological research tool, numerous investigations have been undertaken in tobacco to examine its evolutionary and genomic structure and organization. Wild *Nicotiana* species are store houses of genes for several diseases and pests, in addition to genes for several important quality traits and phytochemicals, which are not present in cultivated varieties. Molecular techniques could play a major role in the confirmation of conventional classification of the genus *Nicotiana*

Molecular genetic markers have become useful tools in providing a relatively unbiased estimation of genetic diversity and phylogeny in plants. PCR based markers like randomly amplified polymorphic DNA (RAPD) are being used in the analysis of genetic diversity in crop plants because of the relative ease with which PCR assays can be carried out as compared to other markers. Besides, prior knowledge about the genome is also not a pre-requisite, which makes RAPD a common method for such studies in different crops. In tobacco, RAPD has been used mainly to identify markers linked to genes for
resistance to pathogens. RAPD procedure was developed for tobacco by using 12 species of *Nicotiana* and 12 varieties of *N. tabacum* using 3 random primers. Evolutionary relationship among the 7 species of *Nicotiana* has been analyzed by amplified fragment length polymorphism (AFLP) markers earlier. However, there are no comprehensive reports on molecular evolutionary relationships among all the major species of *Nicotiana*. This would not only help in understanding the genetic diversity among the species as well as the derived species specific markers but also useful in identification of introgressed interspecific derivatives. This paper reports the results of a study on the genetic diversity based on RAPD among the 22 wild species representing 3 sub genera and 2 interspecific hybrids, which have been used as donors of biotic stress tolerance genes.

**Materials and Methods**

**Plant Material**

The seeds of 22 species and 2 interspecific hybrids of the genus *Nicotiana* were obtained from Central Tobacco Research Institute (CTRI), Rajahmundry, India (Table 1).

**DNA Extraction and RAPD Assay**

Thirty-d-old seedlings of each variety were collected, bulked, frozen in liquid nitrogen, and stored at −80°C until they were used for DNA extraction. DNA was extracted from pulverized frozen seedlings and quantified on 0.8% agarose gel. The following PCR amplification conditions were followed with minor modifications. Amplifications were carried out in a 25 µL reaction mixture containing 15 ng template DNA, 0.5 units of Taq DNA polymerase, 0.2 mM of each dNTP and 20 ng of each primer. Sixty random primers (Operon Technologies, Alameda, CA, USA) were used for amplification. PCR cycles consisted of one cycle at 94°C for 1 min, 37°C for 1 min and 72°C for 2 min followed by 44 cycles of same conditions. Final primer extension for 7 min was carried out at 72°C. Fourteen µL of the amplification product was electrophoresed on 1.2% agarose gel in 1x TBE buffer and stained using ethidium bromide. The size of the fragments was estimated using Gene Ruler 100 bp DNA ladder plus (MBI Fermentas, Lithuania) marker.

<table>
<thead>
<tr>
<th>Subgenus</th>
<th>Section</th>
<th>Species</th>
<th>Somatic chromosome no.</th>
<th>Resistance to diseases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rustica</td>
<td>Paniculatae</td>
<td>glauca</td>
<td>24</td>
<td>PM, An, RV, TE</td>
</tr>
<tr>
<td></td>
<td></td>
<td>knightiana</td>
<td>24</td>
<td>PM, RK, TE</td>
</tr>
<tr>
<td>Tabacum</td>
<td>Tomentosae</td>
<td>glutinosa</td>
<td>24</td>
<td>PM, TMV</td>
</tr>
<tr>
<td></td>
<td>Genuinae</td>
<td>MC12</td>
<td>48</td>
<td>BS</td>
</tr>
<tr>
<td>Petunioideae</td>
<td>Undulatae</td>
<td>undulata</td>
<td>24</td>
<td>RV, TE, TMV, WF</td>
</tr>
<tr>
<td></td>
<td>Trigonophyllae</td>
<td>palmeri</td>
<td>24</td>
<td>An, PM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>trigonophylla</td>
<td>24</td>
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</tr>
<tr>
<td></td>
<td>Alatae</td>
<td>longiflora</td>
<td>20</td>
<td>An, RR, RK, WF, PM</td>
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<td>plumbagenfjolia</td>
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<tr>
<td>Repandae</td>
<td>reبدا</td>
<td>48</td>
<td>An, CN, PM, RK, TMV</td>
<td></td>
</tr>
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<td></td>
<td>nesophila</td>
<td>48</td>
<td>An</td>
<td></td>
</tr>
<tr>
<td></td>
<td>umbratica-nes.</td>
<td>48</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>stocktonii</td>
<td>48</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Nudicaules</td>
<td>nudicaulis</td>
<td>48</td>
<td>An, PM, RK, WF</td>
<td></td>
</tr>
<tr>
<td>Suaveolentes</td>
<td>benthamiana</td>
<td>38</td>
<td>PM, TMV</td>
<td></td>
</tr>
<tr>
<td></td>
<td>benhamiana-reبدا</td>
<td>48</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>gosesi</td>
<td>36</td>
<td>RR, PM, TMV, TS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>amplexicaulis</td>
<td>36</td>
<td>BM, SP, PM, TMV</td>
<td></td>
</tr>
<tr>
<td></td>
<td>velatina</td>
<td>32</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>hesperis</td>
<td>42</td>
<td>BM</td>
<td></td>
</tr>
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<td></td>
<td>occidentales</td>
<td>42</td>
<td>An, SP, PM</td>
<td></td>
</tr>
<tr>
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<td>megalosiphon</td>
<td>40</td>
<td>An, PM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>excelsior</td>
<td>38</td>
<td>PM</td>
<td></td>
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<td></td>
<td>debneyi</td>
<td>48</td>
<td>An, PR, BM, PM, RV</td>
<td></td>
</tr>
<tr>
<td></td>
<td>stenocarpa</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

Data Analysis

Clearly resolved bands were scored for presence (1) or absence (0). The data on 229 bands generated by 18 primers were selected for the analysis of genetic diversity. The NTSYS-pc software version 2.0211 was used to calculate similarity coefficients12. Based on UPGMA and SAHN clustering, a dendrogram depicting the genetic relationship among the varieties was prepared. Mean similarity of individual species with the rest and among the species within a particular cluster was computed from the similarity matrix table. Polymorphism information content (PIC) was calculated using formulae \( PIC = 2xP_iQ_j \), where \( P_i \) is the frequency of the presence of band and \( Q_j \) is the frequency of its absence13. PIC values for all the polyphomorphic fragments for a primer were averaged to provide PIC value for a primer.

Results and Discussion

Level of Polymorphism

Twenty-eight random decamer primers were used in this study, out of which 18 were chosen for analysis based on clear and well resolved RAPD pattern. The number of fragments amplified by each primer ranged between 9-17 with an average of 12.7 fragments/primers across all the three subgenera of the genus Nicotiana. The fragment polymorphism was higher than the reported in case of egg plant (10.28 fragments/primer), which also belongs to the family Solanaeae14. Maximum number of bands (17) was produced by the primer OPP18 whereas minimum number of bands (9) was produced by the primer OPP12. A representative RAPD profile obtained with the primer OPAB1 and OPAC1 is given in Fig. 1.

The primers OPAC10 and OPAC1 produced maximum number of bands (8) in the subgenus Rustica, whereas the primer OPD13 produced maximum number of bands (8 and 16) in Tabacum and Petunioides, respectively (Table 2). The minimum number of bands was observed with the primers OPAC2, OPD11 and OPC5 (2, 1 and 7) in the subgenera Rustica, Tabacum and Petunioides, respectively. The number of fragments amplified in N. tabacum was significantly higher than that reported earlier7.

All the primers except OPC11 were polymorphic. Among the 18 tested primers 77, 7 and 5 showed complete polymorphism in the subgenera Petunioides, Rustica and Tabacum, respectively. The PIC scores per primer across the genus ranged from 0.134-0.366 with an average of 0.274. The primer OPC16 gave highest (0.366) and OPC11 gave the lowest PIC (0.134). Six primers gave PIC value more than 0.3 whereas 12 primers showed PIC value more than the average PIC. The high PIC primers would further be utilized for characterization of species.

A total of 229 bands were amplified of which 228 (99.5%) were polymorphic across the genus Nicotiana. The level of polymorphism among the species of the subgenus Rustica and Tabacum was 74.73% and 76.82% whereas in the species of the subgenus Petunioides much higher level of polymorphism (99.07%) was observed. The subgenus Petunioides is one of the largest genera, which contains species having chromosome numbers between 20 and 48. The high degree of genetic polymorphism among the 7 species of Nicotiana was also reported using AFLP8. In contrast, genetic diversity analysis by RAPD markers revealed 79% polymorphism in the species of L. peruvianum and very low (9%) in the species L. parviflorum of the genus Lycopersicon15.

Species Specific Markers

A total of 39 species-specific markers were generated, of which, 6 were specific to subgenus Rustica, 2 to subgenus Tabacum, and 31 to subgenus Petunioides (Table 3). Since some of the species were crossable with cultivated species, the target genes were being transferred to desirable agronomic base through backcross breeding. The identification of interspecific hybrids and assessment of gene introgression had been based on phenotype that was highly subjective. The species specific markers identified in this study would aid unambiguous identification of the true hybrids, monitoring introgression of the target gene(s) and estimation of the genetic background of the desirable segregants particularly with regard to the genomic contribution of the wild species.

Among the 22 species, 19 were clearly differentiated by producing one or two specific bands with all the 18 primers used (Table 2). The primer OPAC2 gave two specific bands to three species namely; N. triganophylla, N. benthamiana and N. repanda (Table 2). Species-specific RAPD patterns have been developed and used to confirm hybridity in potato16 and intergeneric hybrids of Sacharum and Erainthu17.

Genetic Relationships in Nicotiana

The genetic similarity among the three subgenera of Nicotiana was low (19.2%). However, the percentage
percentage of bands shared between subgenus *Tabacum* and *Rustica* was more (43.9%) which had remained conserved during evolution shows the evolutionary closeness of these two subgenera. Sequence analysis of multiple nuclear fragments from such potentially conserved regions of the genome would be useful in understanding the phylogenetic relationships and characterizing the pattern of divergence. Goodspeed\(^1\) had postulated that the present day assemblage of species was derived from a pregeneric genetic reservoir with three major components that had been designated pre-*Nicotiana*, pre-*Cestrum* and pre-*Petunia*. The Cestroid complex was thought to be ancestral to the subgenera *Tabacum* and *Rustica* whereas *Petuniod* complex ancestral to the subgenus *Petunioides*. Grouping of these species was clearly in accordance with the earlier classification based on traditional analysis of cytological and morphological characteristics\(^1\). The subgenus *Tabacum* shared 34.10% of amplified fragments with *Petunioides* whereas *Rustica* shared more (39.63%) amplified fragments. The subgenus

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**Table 2—Analysis of polymorphism obtained with random primers among various species of genus *Nicotiana* Figures in the parentheses represent % of polymorphism.**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Genus <em>Nicotiana</em></th>
<th>Subgenus <em>Rustica</em></th>
<th>Subgenus <em>Tabacum</em></th>
<th>Subgenus <em>Petunioides</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total number of bands</td>
<td>Number of polymorphic bands</td>
<td>Total number of bands</td>
<td>Number of polymorphic bands</td>
</tr>
<tr>
<td>OPAB1</td>
<td>15</td>
<td>15(100%)</td>
<td>6</td>
<td>4(66.6%)</td>
</tr>
<tr>
<td>OPAC1</td>
<td>14</td>
<td>14(100%)</td>
<td>8</td>
<td>5(62.5%)</td>
</tr>
<tr>
<td>OPC11</td>
<td>10</td>
<td>9(90%)</td>
<td>4</td>
<td>1(25%)</td>
</tr>
<tr>
<td>OPC12</td>
<td>14</td>
<td>14(100%)</td>
<td>4</td>
<td>4(100%)</td>
</tr>
<tr>
<td>OPC13</td>
<td>11</td>
<td>11(100%)</td>
<td>4</td>
<td>4(100%)</td>
</tr>
<tr>
<td>OPC15</td>
<td>11</td>
<td>11(100%)</td>
<td>4</td>
<td>4(100%)</td>
</tr>
<tr>
<td>OPC4</td>
<td>16</td>
<td>16(100%)</td>
<td>7</td>
<td>6(85.7%)</td>
</tr>
<tr>
<td>OPC8</td>
<td>13</td>
<td>13(100%)</td>
<td>5</td>
<td>4(80%)</td>
</tr>
<tr>
<td>OPAB12</td>
<td>15</td>
<td>15(100%)</td>
<td>5</td>
<td>5(100%)</td>
</tr>
<tr>
<td>OPC2</td>
<td>9</td>
<td>9(100%)</td>
<td>2</td>
<td>2(100%)</td>
</tr>
<tr>
<td>OPAC10</td>
<td>14</td>
<td>14(100%)</td>
<td>8</td>
<td>4(50%)</td>
</tr>
<tr>
<td>OPD11</td>
<td>9</td>
<td>9(100%)</td>
<td>4</td>
<td>4(100%)</td>
</tr>
<tr>
<td>OPD18</td>
<td>13</td>
<td>13(100%)</td>
<td>4</td>
<td>3(75%)</td>
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<tr>
<td>OPC16</td>
<td>12</td>
<td>12(100%)</td>
<td>7</td>
<td>6(85%)</td>
</tr>
<tr>
<td>OPC18</td>
<td>13</td>
<td>13(100%)</td>
<td>5</td>
<td>4(80%)</td>
</tr>
<tr>
<td>OPC5</td>
<td>10</td>
<td>10(100%)</td>
<td>6</td>
<td>5(83%)</td>
</tr>
<tr>
<td>OPC14</td>
<td>13</td>
<td>13(100%)</td>
<td>5</td>
<td>5(100%)</td>
</tr>
<tr>
<td>OPD13</td>
<td>17</td>
<td>17(100%)</td>
<td>7</td>
<td>1(14.2%)</td>
</tr>
</tbody>
</table>

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Petunioides is close to the subgenus Rustica because the pre Rustica (pre subgeneric component) contributed to the evolution of species from pre Petunioides (pre subgeneric component).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Marker specific to</th>
<th>Marker specific to</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPAB1</td>
<td>350 bp-N. glutinosa, 2450 bp-N. amplexicaulis</td>
<td></td>
</tr>
<tr>
<td>OPAC1</td>
<td>2250 bp-N. glauca</td>
<td></td>
</tr>
<tr>
<td>OPC11</td>
<td>750 bp-N. glutinosa, 1900 bp, 600 bp-N. megalosiphon, 700 bp-N. undulata</td>
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</tr>
<tr>
<td>OPC12</td>
<td>650 bp-MC12, 820 bp-N. undulata</td>
<td></td>
</tr>
<tr>
<td>OPC13</td>
<td>1020 bp-N. um-nes</td>
<td></td>
</tr>
<tr>
<td>OPC15</td>
<td>1900 bp-N. knightiana</td>
<td></td>
</tr>
<tr>
<td>OPC4</td>
<td>2450 bp-N. hesperis, 820 bp-N. trigonophylla</td>
<td></td>
</tr>
<tr>
<td>OPC8</td>
<td>500 bp-N. glauca, 1600 bp-N. excelsior</td>
<td></td>
</tr>
<tr>
<td>OPAB12</td>
<td>1200 bp-N. knightiana, 2820 bp-N. longiflora, 1250 bp-N. um-nes, 1000 bp-N. ben-rep, 720 bp-N. undulata, 2050 bp-N. ben-rep</td>
<td></td>
</tr>
<tr>
<td>OPAC2</td>
<td>2520 bp-N. trigonophylla, 1400 bp-N. undulata, 800 bp-N. benthamiana</td>
<td></td>
</tr>
<tr>
<td>OPAC10</td>
<td>2820 bp-N. nesophila, 2000 bp-N. longiflora</td>
<td></td>
</tr>
<tr>
<td>OPD11</td>
<td>1800 bp-N. glauca, 1400 bp-N. nesophila</td>
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<tr>
<td>OPD18</td>
<td>750 bp-N. knightiana</td>
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<td>OPC16</td>
<td>2250 bp-N. palmeri</td>
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<tr>
<td>OPC18</td>
<td>2050 bp-N. amplexicaulis, 1450 bp-N. occidentalis</td>
<td></td>
</tr>
<tr>
<td>OPC5</td>
<td>2000 bp-MC12, 1950 bp-N. nudicaulis</td>
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<tr>
<td>OPC14</td>
<td>2820 bp-N. megalosiphon, 1700 bp-N. ben-rep, 900 bp-N. undulata</td>
<td></td>
</tr>
<tr>
<td>OPD13</td>
<td>300 bp-N. glutinosa</td>
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</tr>
</tbody>
</table>

RAPD Based Genetic Relationships

All the species of Nicotiana have been separated into 5 main clusters (1-5) based on subgenera specificity and number of chromosomes (Fig. 2). The pairwise similarity measured in the species of subgenus Rustica was 0.252 whereas in the subgenus Tabacum it was 0.189 suggesting that significant diversity among the species of these subgenera. In the species of subgenus Petunioides, the range of pairwise similarity measured was 0.128 to 0.941, which was due to the high genetic similarity among the species belonging to the sections of Rependae and Trigonophyllae. The 3 species of Rependae and 2 species of Trigonophyllae were very closely related even in morphological as well as cytological characters.

Subgrouping of the species within the main clusters is largely based on the chromosome number and sections of the traditional classification, which they belong to. Two species of Rustica remained together in the cluster-1, both the species had equal chromosomal number (2n=24). In cluster-2, the cultivar of N. tabacum, MC-12 and interspecific hybrid N. benthamiana-repanda which has 48 chromosomes (2n=40) belonging to the subgenus Petunioides was independently linked to this group. The cluster-3 divided into two subclusters (a and b) based on the sections of the traditional classification. The subcluster-a formed with the species rependa, nesophila, and...
stocktonii of section Rependae. The interspecific
derivative N. umbretica × N. nesophila was also
clustered in the same group since nesophila is one of the
parent to this inter specific hybrid, all of them had same
number of chromosomes (2n=48). The species nudicalis
and benthamiana formed a group and linked to the
subcluster-a. In the present study, the species N.
benthamiana was not clustered in 3b along with other
members of section suaveolantes but grouped in cluster
3a along with the N. nudicaulis, the member of section
 nudicaules. Goodspeed1 reported that the correlations
between numerical and morphological variations in the
chromosomes were consistent throughout the section
suaveolantes of subgenus Petunioides with the
exception of 19 paired N. benthamiana. The subcluster–
b formed with 7 species of section suaveolentes. The cluster-4 formed with the species of the section
trigannahyllae and the species of section undulate and
all of these species had 2n=24 chromosomes. The cluster-5 was formed by the two species longiflora and
plumbagenifolia of section alatae, these two species had
2n= 20 chromosomes. The species glutinosa of the
section tomentosae of subgenus Tabacum with 24
chromosomes was not linked to the any of the clusters.
According to Goodspeed1 in the subgenus Tabacum,
section tomentosae consists of 5 species of which
tomentosae, tomentosiformis and otophora are
considered as core group and the other two species,
glutinosa and setchellii are distinct from the core group
due to their morphological and cytological characters,
which may be responsible for the glutinosa to cluster
differently from the subgenus tabacum.

Grouping of species considered under the present study
was in accordance with the traditional classification, which
was mainly based on the chromosome number. Though
the subgenus petunioides consists of species with varied
number of chromosomes, they largely clustered based on
the sections of the taxonomical classification. However,
the RAPD analysis of Carthamus has modified the
existing taxonomic and karyotypic classification19.

In conclusion, the present study would be useful for
establishing molecular phylogeny in the species of
Nicotiana. RAPD assay was also found effective in
analyzing polymorphism at the subspecies level. The
species-specific markers identified would be utilized in
introgression breeding programmes.

Acknowledgement
The authors are highly thankful to the Director,
CTRI, for his encouragement and suggestions during
the course of this study.

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