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ORIGINAL PAPER

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Development of SCAR markers linked to three disease resistances based on AFLP within *Nicotiana tabacum* L

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Abstract Amplified fragment length polymorphism (AFLP) was conducted on a set of 92 *Nicotiana tabacum* L. accessions from diverse types (flue-cured, dark air-cured, burley, oriental, and cigar wrapper) and breeding origins to identify markers associated with disease resistances. Eleven primer combinations were required to identify 33 polymorphic fragments. This allowed the identification of 92% of these accessions, and yielded sufficient information for building a neighbor joining tree. Clusters of accessions with common traits or breeding origins were observed. An important part of this polymorphism could be related to interspecific introgressions from other *Nicotiana* species, performed during the breeding history of *N. tabacum* to confer resistance to pathogens. Seven fragments were associated with three different resistances: two for the blue-mold (*Peronospora tabacina* Adam) resistance derived from *Nicotiana debneyi* Domin, two for the *Va* gene (Potato Virus Y susceptibility), and three for the black root rot (*Chalara elegans*) resistance of *N. debneyi* origin. Some of these markers were converted into sequence characterized amplified region markers, and validated on recombinant inbred lines or doubled-haploid lines.

Keywords Nicotiana tabacum · AFLP · Blue mold · PVY^N · Black root rot · SCAR markers

Introduction

Tobacco, the most important non-food crop grown world-wide (Tso 1990), is also an intensively studied model for plant biology. Commercial tobacco cultivars belong to the species *Nicotiana tabacum*, an allotetraploid ($2n = 48$) thought to have arisen from interspecific hybridization between *Nicotiana sylvestris* ($2n = 24$, sub-genus *Petunioides*, section *Alatae*) and *Nicotiana tomentosiformis* ($2n = 24$, sub-genus *Tabacum*, section *Tomentosae*) (Matassi et al. 1991).

Random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) were readily found among species of the sub-genus *Tabacum*, but most of the corresponding markers failed to show such polymorphism within the species *N. tabacum* (Del Piano et al. 2000; Ren and Timko 2001; Rossi et al. 2001). Using RAPD on *N. tabacum*, the general experience is that less than one polymorphism is revealed for every ten decamer primers tested (Wernsman 1999). Consequently, polymorphic molecular markers within *N. tabacum* are scarce when compared to other plants such as tomato or cowpea (Saliba-Colombani et al. 2000; Ouedraogo et al. 2002). No complete genetic map of the *N. tabacum* genome could be proposed so far.

This apparent lack of molecular diversity could be related to the low genetic diversity that is suspected within the *N. tabacum* germplasm. Tobacco evolved under a highly self-pollinated reproductive mode. Prior to the 20th century, early selection may have led to the differentiation into the main tobacco types, a process during which only a few genotypes became the progenitors of most of the modern cultivars (Wernsman 1999).

A major incentive for creating new *N. tabacum* cultivars came from the need to control epidemic tobacco diseases. Consistent with the low genetic diversity hypothesis, it soon became apparent that only a few resistances to the main diseases could be found within *N. tabacum*.

This situation is illustrated by the blue-mold disease caused by the oomycete *Peronospora tabacina* Adams. Essentially no *N. tabacum* accession was found to be resistant, and considerable effort has been devoted to transferring resistance from several Australian *Nicotiana* species (Rufty 1989). Partially resistant cultivars were developed from two sources: Bel 61-10, which likely

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inherited its resistance from *N. debneyi*, and Ovens 62, derived from crosses involving *Nicotiana goodspeedii* and *Nicotiana velutina* (Wark 1963).

Soil-borne diseases of tobacco are also a major concern, among which is the black root rot disease caused by the fungus *Chalara elegans* [*C. elegans* Nag Raj and Kendrick; syn. *Thielaviopsis basicola* (Berk. and Broome) Ferraris]. The complete immunity to black root rot found in *N. debneyi* was successfully transferred to *N. tabacum* (Clayton 1969) leading to the line Br-RMW.

Another important tobacco pathogen in Europe is the Potato Virus Y necrotic strain (PVY^N). Within *N. tabacum*, partial resistance is conferred by a single recessive factor called *va*, assigned to chromosome E (Gupton and Burk 1973; Yamamoto 1992).

In all the above examples, adverse effects on tobacco leaf quality due to the resistance genes themselves or to linked genetic factors have been reported or suspected (Legg et al. 1981; Wernsman 1999). Genetic markers in close association with resistance genes would provide valuable tools for rapid identification of superior resistant lines with high-quality traits.

Currently, several markers linked to tobacco resistance genes have been proposed. RAPD markers linked to the black root rot resistance factor (Bai et al. 1995), to the "va" gene (Noguchi et al. 1999), or to the "Ph" gene conferring resistance to the black shank disease (Johnson et al. 2002) were obtained. The root-knot nematode resistance gene "Rk" has been mapped using RAPD markers (Yi et al. 1998). A QTL explaining 34% of the variance in resistance to the bacterial wilt disease has been found using AFLP (Nishi et al. 2003).

The majority of these markers were obtained with RAPD, and compared to AFLP; the transfer of this method between laboratories is not always successful (Jones et al. 1997). The goal of this study is to develop reliable markers linked to the resistances to blue mold, black root rot, or PVY^N, the main tobacco diseases in Europe. In order to increase the chances of finding some polymorphism, AFLP was assessed on a set of 92 *N. tabacum* accessions that are particularly representative of the germplasm base for tobacco breeding. This set comprises all tobacco types (flue-cured, burley, dark air-cured, cigar wrapper, oriental), recent and antique cultivars, and is also balanced for the presence or absence of resistance genes to the three target diseases. Association of AFLP markers with one of the three resistance traits was first assessed within the collection, and then validated in specific segregating populations.

Materials and methods

Plant material

The 92 *N. tabacum* accessions included 76 inbred lines maintained in the Altadis-ITB germplasm collection (Delon et al. 1999), and 16 F_1 hybrids representative of cultivars currently grown in France (Table 1). The different tobacco types are represented (flue-cured, burley, dark air-cured, oriental, and cigar wrapper) from several origins. Thirty-one are black root rot resistant, 44 are PVY^N resistant, and 13 are blue-mold resistant.

The first segregating population used consisted in F6 RILs obtained by random single seed descent from the original cross 4K78-5 × ITB32 (Table 1). 4K78-5 is susceptible to PVY^N and black root rot, whereas ITB 32 possesses the "va" gene conferring resistance to PVY^N and the black root rot resistance factor from *N. debneyi* origin. The sequence characterized amplified region (SCAR) markers linked to PVY resistance, and black root rot resistance was assessed on 103 and 109 F6 RILs, respectively.

The second segregating population consisted in doubled-haploid (DH) lines derived from the F_1 BB16 × TN86 (Table 1) using the anther culture method (Nakamura et al. 1974). While TN86 is susceptible to *P. tabacina*, BB16 possesses the resistance factors inherited from Bel 61-10. As a preliminary experiment, the SCAR marker associated with *P. tabacina* resistance was assessed on 17 DH lines.

Evaluation of pathogen resistance

PVY^N resistance

Leaves from a susceptible (*VaVa*) plant infected with a PVY^N strain belonging to pathotype 2 (Blancard et al. 1995) were crushed in a mortar. For 1 g of fresh leaf, the sap was diluted into 4 ml of Na_2HPO_4 (0.3 mol/l) + DIECA (2 g/l) + 0.3 mg carborundum. This inoculum was gently rubbed on two leaves of 4-week-old tobacco plants grown in a temperature-controlled greenhouse (18–23°C) under natural light. For each inbred line, 12 plants were inoculated. Approximately 3 weeks after inoculation, mosaic (vein clearing, vein yellowing, vein mottling, or chlorotic spots) and necrotic (vein necrosis, stalk necrosis) symptoms were examined. Resistant plants do not express necrotic symptoms in these conditions, but may display some mosaic symptoms. The number of plants expressing necrotic symptoms was counted. Lines were assumed to be resistant when none of the plants expressed necrotic symptoms, susceptible when every plant expressed necrotic symptoms, and otherwise segregating. Resistant (PBD6, ITB32, TN86) and susceptible (BB16, ITB30, 4K78) controls were replicated at regular intervals within the tests to allow for visual comparison.

Peronospora tabacina resistance

After growth in a pathogen-free environment, 5-week-old plants were transplanted into a ventilated greenhouse, approximately 4 weeks before climatic conditions became favorable to *P. tabacina*, with

Table 1 *N. tabacum* lines and hybrids, classified as determined in Fig. 1

No.	Cluster	Name	Type	Hybrid/line	TD[a]	va va[b]	PB[c]	PO[d]	Origin[e]
8	1	Maryland 872	DAC	Line	0	0	0	0	U.S.A.
2	1a	Bel 61-10	DAC	Line	0	0	1	0	Beltsville
10	1a	PBD6	DAC	Line	0	1	1	0	ITB
11	1a	ITB1000	DAC	F1 hybrid	0	1	1	0	ITB
12	1a	ITBBR3	DAC	F1 hybrid	1	1	1	0	ITB
14	1a	ITBBR5	DAC	F1 hybrid	0	0	1	0	ITB
16	1a	LAP41	DAC	Line	0	1	1	0	ITB
18	1a	BB16	Bey	Line	0	0	1	0	ITB
19	1a	BB16NN	Bey	Line	0	0	1	0	ITB
41	1a	LAP57	Bey	Line	0	1	0	0	ITB
91	1a	Perevi	FC	Line	0	1	1	0	LFPF
17	1b	Chemical Mutant	DAC	Line	0	0	0	0	Australia
20	1b	BY21	Bey	Line	0	0	0	0	Beltsville
21	1b	BY21 LA	Bey	Line	0	0	0	0	Ky
56	1b	BRV31	FC	Line	0				ISSPC
3	2	Criollo Correntino	DAC	Line	0	0	0	0	Argentina
71	2	Samsoun	OR	Line	0		0	0	Turkey
72	2	Samsoun H	OR	Line	0		0	0	Turkey
73	2	Xanthi	OR	Line	0		0	0	Greece
74	2	Basma Drama	OR	Line	0		0	0	Greece
75	2	Izmir	OR	Line	0		0	0	Turkey
76	2	Meteores	OR	Line	0		0	0	Greece
77	2	Kabakoulak	OR	Line	0		0	0	Greece
78	2	Xanthi NC	OR	Line	0		0	0	Greece
79	2	BelB	CW	Line	0		0	0	Beltsville
80	2	BelW3	CW	Line	0		0	0	Beltsville
81	2	Havana Cubano Q	DAC	Line	0		0	0	Carribean
27	3	White Burley	Bey	Line	0	0	0	0	INAC
90	3	Delgold	FC	Line	0	0	0	0	AC
40	3a	G94-2	Bey	Line	0	1			IST
60	3a	K326	FC	Line	0	0	0	0	GL
61	3a	K394	FC	Line	0	0	0	0	GL
62	3a	NC 95	FC	Line	0	0	0	0	NCSU
63	3a	NC 2326	FC	Line	0	0	0	0	NCSU
64	3a	K399	FC	Line	0	0	0	0	GL
65	3a	MN944	FC	Line	0	0	0	0	U.S.A.
66	3a	Kutsaga 51E	FC	Line	0	0	0	0	Zim
67	3a	Coker 86	FC	Line	0	0	0	0	U.S.A.
69	3a	Speight G28	FC	Line	0	0	0	0	SP
84	3a	LAFC53	FC	Line	0	0	0	0	Zim
92	3a	4K78	FC	Line	0	0	0	0	ITB
48	3b	ITB30	FC	Line	0	0	0	0	ITB
49	3b	ITB3413	FC	F1 hybrid	0	0	0	0	ITB
59	3b	ITBVIR17	FC	F1 hybrid	0	0	0	0	ITB
68	3b	NC13	FC	Line	0	0	0	0	U.S.A.
83	3b	TB22	FC	Line	0	0	0	0	Zim
89	3b	Islangold	FC	Line	0	0	0	0	AC
26	4a	ITB44-90	Bey	Line	1	1	0	0	ITB
30	4a	ITB501	Bey	F1 hybrid	1	1	0	0	ITB
31	4a	ITB502	Bey	F1 hybrid	1	1	0	0	ITB
32	4a	ITB503	Bey	F1 hybrid	1	1	0	0	ITB
33	4a	ITB509	Bey	F1 hybrid	1	1	1	0	ITB
35	4a	ITB2604	Bey	F1 hybrid	1	1	0	0	ITB
36	4a	ITB218	Bey	Line	1	1	0	0	ITB
37	4a	ITBBY10	Bey	Line	1	1	0	0	ITB
38	4a	TN86	Bey	Line	1	1	0	0	TN
39	4a	TN90	Bey	Line	1	1	0	0	TN
42	4a	ITBBY11	Bey	Line	1	1	1	0	ITB
43	4a	ITBBY12	Bey	Line	1	1	0	0	ITB
86	4a	Vty8G1	FC	Line	1	1	0	0	ITB
4	4b	Kentucky170	DAC	Line	1	0	0	0	Ky
5	4b	Kentucky170R	DAC	Line	1	0	0	0	Ky
23	4b	Kentucky17	Bey	Line	1	0	0	0	Ky
34	4b	ITB2204	Bey	F1 hybrid	1	0	0	0	ITB
44	4c	ITB3304	FC	F1 hybrid	1	1	0	0	ITB
46	4c	ITB30804	FC	F1 hybrid	1	1	0	0	ITB
47	4c	ITB30808	FC	F1 hybrid	1	1	0	0	ITB

Table 1 (Contd.)

No.	Cluster	Name	Type	Hybrid/line	TD[a]	va va[b]	PB[c]	PO[d]	Origin[e]
50	4c	ITB620	FC	F1 hybrid	1	1	0	0	ITB
52	4c	ITBVIR12	FC	Line	1	1	0	0	ITB
53	4c	ITBVIR13	FC	Line	1	1	0	0	ITB
54	4c	ITB32	FC	Line	1	1	0	0	ITB
57	4c	ITBVIR15	FC	Line	1	1	0	0	ITB
58	4c	ITBVIR16	FC	F1 hybrid	1	1	0	0	ITB
82	4c	ITBVIR11	FC	Line	1	1	0	0	ITB
85	4c	ITBVIR18	FC	Line	1	1	0	0	ITB
87	4d	76C16	FC	Line	1	0	0	0	AC
88	4d	72C18	FC	Line	1	0	0	0	AC
1	5a	Paraguay 48	DAC	Line	0	1	0	0	France (ITB)
6	5a	PS101	DAC	Line	0	1	0	0	France (ITB)
7	5a	PS102	DAC	Line	0	1	0	0	France (ITB)
9	5a	Dragon Vert	DAC	Line	0	1	0	0	France (ITB)
13	5a	ITBBR4	DAC	Line	0	1	0	0	ITB
15	5a	ITBBR8	DAC	Line	0	1	0	0	ITB
25	5a	Virginie A Mutant	Bey	Line	0	1	0	0	Germany (LFPF)
28	5a	Mont Calme Jaune	Bey	Line	0	1	0	0	Switzerland
29	5a	Paesana	Bey	Line	0		0	0	Switzerland
45	5a	VD	FC	Line	0	1	0	0	Germany (LFPF)
22	5b	Bursan	Bey	Line	0	1			Poland (ISSPC)
24	5b	Bursanica	Bey	Line	0	1			Germany (LFPF)
51	5b	VZ37-1-9	FC	Line	0	1	0		ITB
55	5b	Wislica	FC	Line	0	1	0	1	Poland (ISSPC)
70	5b	Ovens 62	FC	Line	0	0	0	1	Australia

DAC dark air-cured, *Bey* burley, *FC* flue-cured, *CW* cigar wrapper, *OR* oriental
[a]TD, *Thielaviopsis basicola* resistance inherited from *N. debneyi*: 1 = resistance, 0 = no resistance
[b]va va, homozygous for an allele of the va gene conferring resistance to PVY^N: 1 = resistance (va va), 0 = no resistance (Va va) or (Va Va)
[c]PB, *Peronospora tabacina* resistance inherited from Bel 61-10 or Bel 61-12: 1 = resistance, 0 = no resistance
[d]PO, *Peronospora tabacina* resistance inherited from Ovens 62: 1 = resistance, 0 = no resistance
[e]AC, Agriculture Canada Res. Station, Delhi, Ontario, Canada; Beltsville, Beltsville Ag. Res. Station, Crops Res. Div, USDA, Beltsville, Maryland, U.S.A.; GL, Gold Leaf Seed Co., U.S.A.; ISSPC, Institute for Soil Science and Plant Conservation, Pulawy, Poland; IST, Istituto Sperimentale Tabacco, Scafati, Italy; ITB, ALTADIS, Institut du tabac, Bergerac, France; Ky, Kentucky University, Lexington, KY, U.S.A; LFPF, Landesanstalt Für Pflazenbau, Forchheim, Germany; NCSU, North Carolina State University, Raleigh, U.S.A.; SP, Speight Seed Farm, U.S.A.; TN, Univ. of Tennessee, TN, U.S.A.; Zim, Tobacco Res. Board, Harare, Zimbabwe

temperatures ranging between 18 and 25°C. High relative humidity could be maintained with the use of an automated irrigation system. A natural *P. tabacina* epidemic occurred, and plants were individually scored for resistance using a scale from 0 (resistant) to 9 (highly susceptible), based on visual assessment of the leaf surface affected by chlorotic spots due to colonization by the pathogen. For evaluating the 17 DH lines, as well as resistant (Bel 61-10, Ovens 62) and susceptible (P48) controls, three replicates of five individual plants each were grown.

Black root rot resistance

A virulent culture of *C. elegans* isolated from tobacco plants grown in France was used as the inoculum source. Petri dishes (50 mm diameter) were filled with a substrate made from an inoculum solution (1×10^6 conidia/ml) mixed with silica (60 ml/100 g). Fifty seeds were germinated for each inbred line. Controlled conditions were applied for germination (14 h of light at 26°C, 10 h of night at 23°C, for 8 days) and growth (14°h of light at 21°C, 10°h of night at 16°C, for 10 days). At 18 days, seedlings were examined under a binocular microscope (40×). In these conditions, plants harboring the *N. debneyi* resistance can be easily recognized due to the absence of black lesions on roots, with no visible chlamydospores. Visual examination of all young plants allows a direct assignment of lines to one of the three categories: resistant, segregating, or susceptible. Resistant (ITB32, TN86) and susceptible (4K78, BB16) controls were introduced in each test.

Markers development and analysis

DNA extraction

Total genomic DNA was isolated from 100 mg of leaf material with a DNeasy Plant Mini Kit from Qiagen (GmbH, Germany). DNA concentration and purity were estimated spectrophotometrically at 260 nm (GeneQuant II, Pharmacia Biotech, Cambridge, UK).

AFLP analysis

Amplified fragment length polymorphism (Vos et al. 1995) was performed with the AFLP Analysis System I

Kit (Invitrogen, Carlsbad, CA, USA) with some modifications: restriction enzyme digestion was performed at 37°C overnight. In the selective amplifications, *Eco*RI primers were 5′ end-labeled with 6-FAM, TET, or NED fluorochromes. Samples were analyzed via capillary electrophoresis on an ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and data were treated with Genescan software. AFLP fragments were treated as separate characters and scored for presence (1) or absence (0) of polymorphic bands in each of the 92 entries. Fragment sizes smaller than 60 bp were less reproducible, and were therefore omitted from the analysis.

Gel electrophoresis for AFLP markers isolation

Amplification products were mixed with an equal volume of loading buffer denatured and analyzed on a 6% denaturing polyacrylamide gel at constant power (55 W, 2,500 V, 50 mA) for 2 h. The gel was stained with the Silver Sequence DNA Sequencing System from Promega (Madison, WI).

Generation of SCAR markers

Amplified fragment length polymorphism fragments of interest were cut from the polyacrylamide gel, and the piece of gel was used as a template for PCR. The size of amplification products was checked on a 1.8% agarose gel. The DNA band was cut from the agarose gel, purified using the Gel Agarose Purification Kit from Qiagen, and cloned into the pCR4-TOPO Vector (Invitrogen, Carlsbad, CA, USA, TOPO TA Cloning Kit for Sequencing). Plasmids were purified with Qiaprep-spin Plasmid Miniprep Kit from Qiagen. Sequencing reactions were performed using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit v1.1 (Applied Biosystems). The sequencing reaction was purified by ethanol precipitation and resuspended in formamide. Sequences were read on ABI 310 Genetic Analyzer (Applied Biosystems) with Sequencing Analysis software. Specific primers were designed from the sequence of each AFLP fragment with Primer Express software (Applied Biosystems). SCAR PCR consisted of 35 cycles of 30 s at 94°C, 45 s at melting temperature, and 1 min at 72°C, with a final extension at 72°C for 5 min.

Data analysis

Neighbor joining tree building

Genetic distances were calculated using the software FreeTree (Pavlicek et al. 1999, available via http://www.natur.cuni.cz/~flegr/freetree.htm) using the Sokal and Sneath 3 coefficient (Sneath and Sokal 1973). Cluster analysis and elaboration of dendrogram showing phenetic relationships between individuals were performed using the neighbor joining tree construction method (Saitou and Nei 1987). Dendrograms were viewed in Tree View 1.5 (Page 1996, available via http://taxonomy.zoology.gla.ac.uk/rod/rod.html). We used OTU (Operational Taxonomic Units)-based jackknifing, which differs from standard jackknifing by random skipping of taxa instead of skipping fragments. The result of the method determines whether skipping of some taxon can change the topology of other branches. The number of skipped taxa in OTU-based jackknifing was fixed to 5, with 1,000 resamplings.

Association between AFLP candidate markers and the resistances

In order to test the hypothesis of gametic disequilibrium at two loci, only the 76 inbred lines have been considered. Data concerning presence–absence of a major disease-resistance gene are those found in Table 1. The chi-square statistic (Weir 1996) was computed using the software STATGRAPHICS Plus 5.0 (©Statistical Graphics Corp. available via http://www.sigmaplus.fr).

Resistance tests and segregation distortion analysis

For blue mold, individual plant scores were subjected to general linear regression on the following model:

Table 2 Markers selected for the genetic diversity study and size of polymorphic fragments

Name[a]	Size of marker (bp)	Name[a]	Size of marker (bp)	Name[a]	Size of marker (bp)
ACG/CAG.3	103	AAG/CAA.6	225	AAG/CAG.7	177
ACG/CAG.4	162	AAG/CAA.7	246	AGG/CAC.4	119
AGG/CAA.3	71	AAG/CAA.8	258	AGG/CAC.6	126
AGG/CAA.4	77	ACC/CAA.1	65	AGG/CAC.7	174
AGG/CAA.5	95	ACC/CAA.3	87	AGG/CAC.8	203
AGG/CAA.6	127	ACC/CAA.4	143	ACA/CTA.2	171
AGG/CAA.7	275	ACC/CAA.5	192	ACA/CTA.3	187
AGG/CAA.8	330	ACC/CAA.6	207	ACC/CTA.1	176
AAG/CAA.3	102	ACC/CAA.7	326	AAG/CAC.1	182
AAG/CAA.4	112	AAG/CAG.5	126	AGG/CTC.2	360
AAG/CAA.5	138	AAG/CAG.6	171	ACG/CTT.1	139

[a]Combination of *Eco*RI primer selective bases followed by MseI primer selective bases

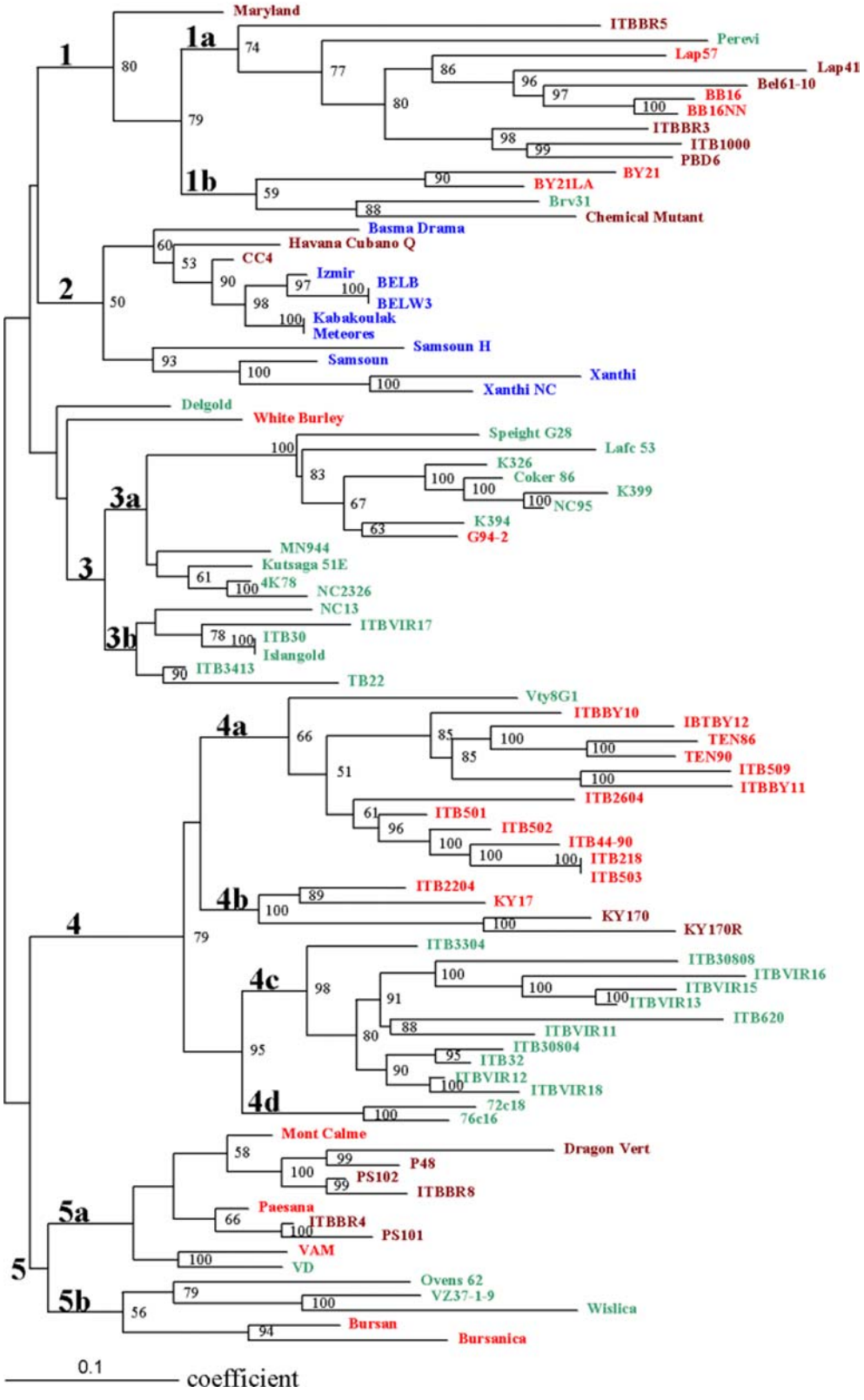

Fig. 1 Dendrogram showing the phenetic relationships among 92 accessions and lines of tobacco (*N. tabacum*). Cluster analysis was performed using the neighbor joining (NJ) method. The resampling method used was OTU-based jackknifing (only values greater than 50 are given at the branch points). The different types of tobacco are represented as indicated: dark air-cured types in *brown*, burley types in *red*, flue-cured types in *green*, and oriental and cigar wrapper types in *blue*

Score = constant term + block effect + SCAR marker effect + DH line (nested within SCAR marker) effect + residual (STATGRAPHICS Plus 5.0).

Chi-square tests were used in analyses of the inheritance of resistances, the SCAR markers, and their linkage. Segregating lines were discarded from the

SCAR marker analysis for black root rot and PVY^N analysis.

Linkage analysis

The genetic linkage between SCAR markers and disease-resistance trait as well as the genetic distances was assessed by Mapmaker 3.0b software (Lander et al. 1987). The linkage was considered significant with an LOD score value above 5.0 and a distance below 40 cM. Recombination fractions were converted into Kosambi centimorgans (cM).

Results

Evaluation of AFLP primer combinations

Preliminary AFLP assays were performed on three lines: PBD6 (dark air-cured type), ITB32 (flue-cured type), and BB16 (burley type). All 64 primer combinations provided in the Invitrogen AFLP Kit were tested. An average of 48 fragments was produced per primer combination, with sizes ranging from 80 to 450 bp. Forty-one combinations generated polymorphic fragments, from one to six per combination. A total of 77 fragments out of 1,970 (3.4%) were polymorphic. Polymorphism ranged from 1.5 to 12.1%, depending on primer combination. Primers E-AAG and E-AGG combined with the eight M-CXX primers produced half of the polymorphic fragments (38 markers out of 77). These preliminary assays allowed us to determine the most efficient primer combinations: E-AGG/M-CAA, E-AGG/M-CAC, E-AGG/M-CTC, E-AAG/M-CAA, E-AAG/M-CAG, E-AAG/M-CAC, E-ACG/M-CAG, E-ACG/M-CTT, E-ACC/M-CAA, E-ACC/M-CTA, E-ACA/M-CTA.

Analysis of the AFLP

The 11 most informative AFLP combinations were used to analyze the genetic diversity of the 92 tobacco accessions. A total of 524 fragments were amplified, of which 6.3% were polymorphic. An average of three polymorphic markers per primer combination was obtained. Thirty-three polymorphic markers were selected among the most reliable (Table 2). The size of polymorphic fragments ranged from 65 to 360 bp. In four cases, two accessions showed the same profile: BelB and BelW3, ITB503 and ITB218, ITB30 and Islangold, and Meteores and Kabakoulak, respectively.

Construction of the NJ tree illustrated in Fig. 1 showed five main clusters.

A clear relationship between the structure of the NJ tree and disease resistances or tobacco types could be observed (Fig. 1 and Table 1). Blue-mold resistant entries were found mainly in clusters 1a (resistance from Bel 61-10), and 5b (resistance from Ovens 62). All black root rot resistant entries (resistance from *N. debneyi* through Br-RMW) were found in cluster 4, the only exception being ITBBR3, found in cluster 1a. PVY^N resistant entries, homozygous for the "va" factor, appeared mainly in clusters 1a, 4, and 5.

Oriental tobacco types were grouped exclusively in cluster 2. Dark air-cured and burley were found in dif-

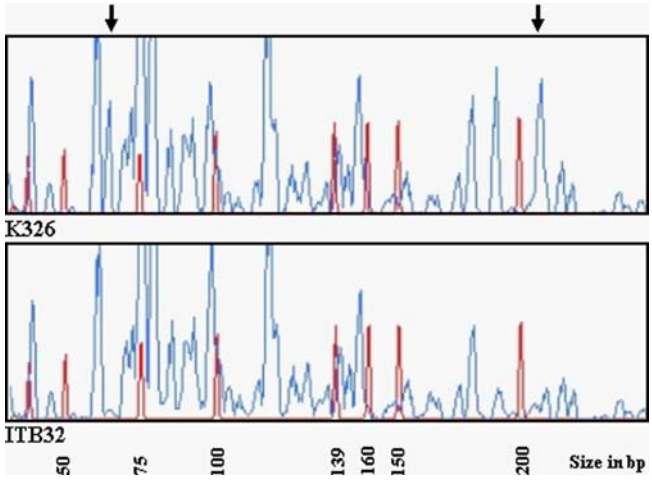

Fig. 2 AFLP peak patterns generated from two tobacco varieties with primer combination E-ACC M-CAA. Tobacco AFLP fragments are represented by *blue peaks*. *Red peaks* represent the size standard, TAMRA 500. Sizes in bp are indicated at the bottom of the figure. The 65-bp fragment (E-ACC M-CAA.1) and the 207-bp fragment (E-ACC M-CAA.6) are amplified in the PVY^N susceptible cultivar (K326) but not in the resistant cultivar (ITB32), and are indicated with *arrows*

Table 3 Association analysis between candidate AFLP markers linked to the resistance to blue mold, black root rot, or PVY

Candidate marker	Linked resistance	Size of AFLP fragment (bp)	Chi-square test for independence[a]	Mismatch between amplification of the marker and resistance in inbred lines
ACG/CAG.4	Black root rot	162	62.9	LAP41, Bursanica
AAG/CAA.3	Black root rot	102	71.9	
AAG/CAG.5	Black root rot	126	71.9	
AGG/CAA.7	Blue mold	275	55.5	LAFC53, Perevi
AGG/CAC.8	Blue mold	203	57.0	LAP57, Xanthi
ACC/CAA.1	PVY	65	45.2	LAFC53, G94-2, By21, Bursan, BRV31, Delgold
ACC/CAA.6	PVY	207	59.3	BRV31, G94-2

[a]Chi-square test for independence between AFLP markers and resistances, with Yates correction

ferent clusters. Cluster 3, however, was mostly flue-cured. Subdivisions of cluster 4 were matching combinations of tobacco types and reaction to PVY^N: 4a and 4b grouped burley types, PVY^N resistant in 4a, and susceptible in 4b. Similarly, 4c and 4d were made of flue-cured entries, PVY^N resistant in 4c, and susceptible in 4d.

These results prompted us to study the degree of information that each marker carried with respect to tobacco type or disease resistance. No tobacco type could be identified by a single marker. However, some types contained some markers at higher frequency. For example, AGG/CAA.6 was found in all dark air-cured and oriental types, but not in burley and flue-cured types. ACC/CAA.5 was found only in flue-cured types, although some flue-cured types lacked it. On the contrary, ACA/CTA.2 and ACC/CTA.1 were often absent in flue-cured types. ACC/CTA.1 was present in all dark air-cured, burley, and oriental types. Some markers were specific to a limited number of cultivars: AGG/CAA.5 was specific to BY21. AAG/CAA.6 was specific to Xanthi and Xanthi NC. ACC/CAA.3 was specific to Samsoun H.

There was a high degree of coincidence between some markers and disease resistances (Table 3). This involved 7 markers out of the 33. Three markers showed some coincidence with the black root rot resistance of *N. debneyi* origin (ACG/CAG.4, AAG/CAA.3, and AAG/CAG.5), two with the *Va* gene conferring PVY^N susceptibility (ACC/CAA.1 and ACC/CAA.6, Fig. 2), and two with the blue-mold resistance (AGG/CAA.7 and AGG/CAC.8). The most coincident AFLP fragments could be cloned to generate SCAR markers: AAG/CAG.5, AGG/CAA.7, and ACC/CAA.6.

SCAR marker linked to black root rot resistance

A SCAR marker derived from the AAG/CAG.5 fragment, Cha11-2, was obtained. Primers designed for direct PCR amplification are given in Table 4. Among the 109 RILs, 71 were assessed as susceptible, 3 as segregating, and only 35 as resistant, showing a segregation distortion for the black root rot resistance factor (Table 5). The SCAR marker was found in all resistant and in only one susceptible line (Table 6). The genetic distance between Cha11-2 and the resistance factor was assessed to be 1 cM, but this could be underestimated due to the segregation distortion.

SCAR marker linked to blue-mold resistance

AGG/CAA.7 has been found indifferently in lines inheriting *P. tabacina* resistance from Ovens 62 or from Bel 61-10. A SCAR marker, Mil275, was obtained (Table 4). Among the 17 DH lines derived from the F_1 cross BB16 × TN86, 11 expressed a high level of resistance (blue mold score < 3), comparable to the resistant control Bel61-10, and the remaining six displayed various degrees of susceptibility (Fig. 3). Mil275 could only be amplified in resistant lines. The presence of the marker is closely related to the blue-mold scores (*F*-test significant at the 0.00001 type I risk level), but statistically significant differences for the reaction to *P. tabacina* also existed between DH lines that do not possess the marker.

Results of this preliminary experiment suggested a link between the SCAR marker and a major QTL for resistance, and also suggested that additional genetic factors modulated the expression of susceptibility.

SCAR marker linked to PVY susceptibility

A SCAR marker derived from ACC/CAA.6, PVYME1, was obtained (Table 4). In the 103 RILs, the *va* gene segregated according to Mendelian rules (54 were resistant, 5 were segregating, and 44 were susceptible, Table 5). No segregation distortion was observed for the SCAR marker. Independence between PVYME1 and *Va* was rejected by the chi-square test (Table 6). Presence of recombinants, however, suggested that the marker was not closely linked with the *Va* locus. The distance between PVYME1 and *Va* was estimated as being 5.1 cM.

Discussion

Breeding origins and population structure

Breeding programs for combinations of disease resistances used some of the 92 studied accessions. Consequently, links exist between disease-resistance traits and genetic backgrounds in each of the five clusters.

In cluster 1, 11 of the 15 entries share lines Bel 61-10, Burley 21, and the cultivar Paraguay in their pedigree. In cluster 2, 8 of the 12 accessions are inbred lines obtained by selfing open-pollinated oriental varieties from Greece or Turkey, likely to come from a common genetic background. Cluster 3 groups flue-cured cultivars from different breeding programs (Table 1, bottom), but the exchange of material between these programs has been intense. Similarly, a substantial number of entries in cluster 4 comes from the Altadis-ITB breeding program developed for the European crop. Finally, in cluster 5, most entries are lines obtained from antique open-pollinated cultivars of European origin (Table 1).

Due to this, direct association tests to reveal linkage between the resistance factors and AFLP markers could have been misleading. Statistical methods to account for the structure of the population (Thornsberry et al. 2001) could be used to resolve this difficulty. However, the relatively low number of polymorphic markers (33), and the fact that 7 of them were highly coincident with diseases resistances, prompted us to check the genetic links through the use of RIL or DH lines. This approach could only be achieved with the SCAR markers on the available populations of DH or RIL lines.

Table 4 Primers used for direct amplification of SCAR markers

Marker	Primers	Sequence	T_m	Length (bp)
Chal1-2	Chal-1	5′-TAACAGCCTAACCCTATTCC-3′	62°C	103
	Chal-2	5′-AATTCAAGGGGTAAAGCTATTC-3′		
Mil27	Mil275-3.1	5′-GCGTGGTTCTTTGTGCGTAT-3′	58°C	205
	Mil275-2.2	5′-TGGGTGTGGAGATAATCAAGC-3′		
PVYME1	PVYM	5′-TTAACAACAGCTTTTAGCAGACAC-3′	62°C	172
	E1	5′-ACAACTGGCAAGCTAAGCTCATT-3′		

Table 5 Segregation distortion tests for PVY^N and black root rot resistances genes, and SCAR markers

Locus	Number of RILs tested	Observed R:S(:Seg)[a]	Expected	Chi-square test for segregation distortion[b]
Black root rot test	109	35:71(:3)	1:1	14.3
Chal1-2	106	35:71	1:1	14.2
PVY test	103	54:44(:5)	1:1	1
PVYME1	98	50:48	1:1	0.04

[a]R for resistant, S for susceptible, (:Seg) for segregants, they were discarded from SCAR marker analysis
[b]3.8 = Chi-square at $P < 0.05$ and one DDL

Table 6 Independence tests between SCAR markers and resistance genes in the F6 RILs

Association	Number of RILs tested	Observed[a]	Expected	Chi-square test for independence[b]
Black root rot test/Chal1-2	106	70[S0]1[R0]1[S1]34[R1]	1:1 ([S0]:[R1])	92.9
PVY test/Pvyme1	98	47[S1]7[S0]3[R1]41[R0]	1:1 ([S1]:[R0])	59.3

[a]R for resistant, S for susceptible, 0 for absence of the SCAR marker, and 1 for presence of the SCAR marker
[b]Chi-square test for independence with Yates correction

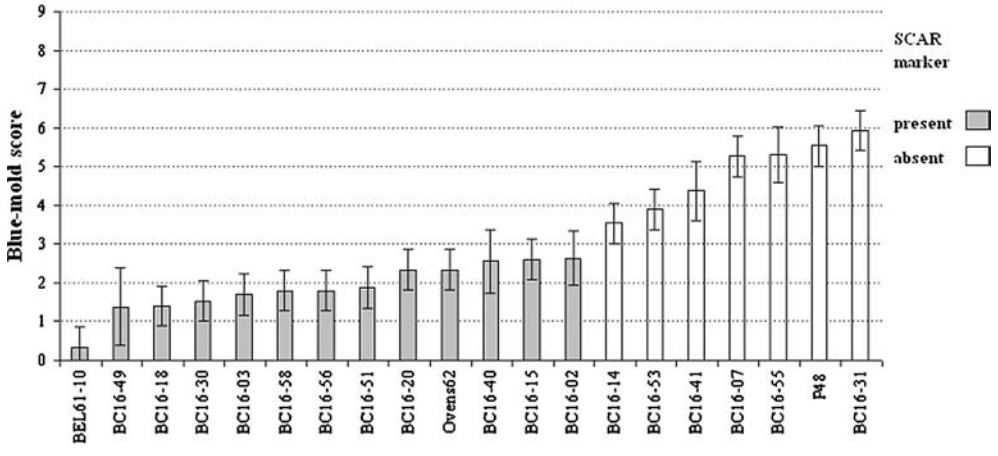

Fig. 3 Blue-mold resistance in 17 DH lines and resistant/susceptible controls. DH lines came from the F_1 BB16 (Bel61-10 resistance) × TN86 (susceptible). P48 is the susceptible control, Bel 61-10 and Ovens 62 are resistant controls. Numbers in the horizontal axis refer to each of the DH lines. *Error bars* display the 95% confidence intervals obtained from the statistical treatment of individual plant data. Each entry was tested with three replications of five plants each. *Gray boxes* indicate DH lines that possess the SCAR marker

AFLP and *Nicotiana debneyi* derived disease resistances

Detailed origins of some lines strongly suggest that *N. debneyi*-derived disease resistances are the primary determinants of clusters 1 and 4, rather than the breeding origins. A common breeding approach is to introgress a disease resistance factor from a donor into a recipient line, in view of obtaining a new resistant line with a phenotype as similar as possible to the recipient. In all examples using *N. debneyi*-derived resistances, the

new line has been classified into the same cluster as the donor, and the recipient appears in another cluster. Resistances derived from *N. debneyi* seem therefore linked to DNA fragments that generate a significant part of the AFLP considered here, with a minimal impact on phenotypic traits that are not related to disease resistances.

Concerning the black root rot disease, resistant cultivars TN86 and TN90 originate from one back-cross to the susceptible line Burley 21 (Miller 1991). Despite their similarity to Burley 21, they are classified in sub-cluster 4a, whereas Burley 21 appears in 1b. Similarly, the resistant cultivar ITB 32 comes from 72C18 (resistant) × VD (susceptible), and appears in cluster 4, close to 72C18, whereas VD is in 5a. In a further breeding cycle, ITB32 was crossed to lines from cluster 3 (K399, K326, ...), which present desirable traits but lack the resistance. Resistant cultivars (ITB 620, ITB 30808, ...) with desirable phenotypes were obtained, but irrespective of their phenotype, they all appear in cluster 4, together with the resistant donor ITB32.

Parallel observations can be made for the blue-mold resistance: PBD6 comes from Paraguay P48 (susceptible) × Bel 61-10 (resistant). Considerable care has been devoted to obtaining a similar phenotype to Paraguay P48 that expresses the blue-mold resistance from Bel61-10 (Schiltz 1967). Despite this, PBD6 appears close to the resistant donor Bel 61-10 in cluster 1a, whereas P48 is in 5a. The same occurs with the flue-cured cultivar Perevi, which originates from VD (susceptible) × Bel 61-12 (resistant), a sister line to Bel 61-10. Despite phenotypic similarity to VD, Perevi appears in 1a with Bel 61-10, whereas VD is in 5a.

Introgressions bearing blue-mold and black root rot resistances were obtained in separate breeding programs. Data suggest that they have affected independent parts of the genome, thus creating separate clusters in the NJ tree. Pyramiding both resistances in the same genotypes could be achieved by using classical breeding methods, as illustrated by the recent cultivars ITB BY11, ITB 509, and ITB BR3.

In the particular case of the black root rot resistance, increased polymorphism due to the high diversity of DNA sequences between the alien transfer and the corresponding host chromosomal segment have been reported (Bai et al. 1995). Another observation that fits with this model is the segregation distortion, in favor of the *N. tabacum* segment, conferring susceptibility that was observed within the RILs.

Concerning the blue-mold resistance, no such distortion could be observed. Recent results showed Mendelian segregations for the SCAR marker Mil275 (data not shown). A higher degree of integration into the *N. tabacum* genome than for the black root rot resistance may therefore be hypothesized.

In a former work (Milla 1998), most of the RAPD markers linked to the Ovens 62 (*N. goodspeedi* origin) resistance were also present in Bel 61-10 (*N. debneyi* origin). This study comes to a similar result. It has been suggested previously that a single gene conditioning adult plant resistance to blue mold had been incorporated at the same *N. tabacum* chromosomal position, after introduction from either *N. debneyi* or *N. goodspeedi* (Wark 1963). To confirm the *N. debneyi* origin of the marker, AFLP and SCAR markers were assessed on one accession of *N. debneyi* (data not shown). The AFLP marker was not amplified, but the SCAR marker was present, which may be consistent with the *N. debneyi* origin or the marker. Some minor modifications in the introgressed sequence could have altered enzyme specific recognition site in the AFLP method.

AFLP and tobacco types

Evidence of separation between types is provided by this study. Cluster 2 groups all oriental types studied here. When considering burley and flue-cured, there is some degree of separation, but no complete congruence with clusters of the NJ tree. This may be due to disease resistances, closely related to seven markers, and which have been introgressed into both of these types. Therefore, separation between these types is clearer when considering a homogeneous set of accessions for introgressed resistances, as can be seen in cluster 4.

The fact that cluster 2 also harbors some dark types or cigar wrapper types (Bel B, Bel W3, Habana Cubano Q, CC4 = Criollo Correntino) could be related to common morphological and physiological characteristics (plant shape, high trichome secretions with the presence of methyl-valeric acids that are absent or lower in burley and flue-cured), and raises the question of a possible common origin. Interestingly, in an earlier study Ren and Timko (2001) also found some degree of relation between oriental accessions and dark types.

Efficiency of AFLP in identifying markers using a diversified set of *Nicotiana tabacum* accessions

Markers linked to three disease resistances originating from introgressions or from deletions (PVY^N resistance: Noguchi et al. 1999) were simultaneously detected by AFLP. Eleven primer combinations identified 33 polymorphic fragments, among which 7 were linked to three different disease resistances. Three were successfully converted into SCAR markers. Linkage between SCAR markers and PVY and black root rot resistance genes could be validated in RILs populations. This may be related to the occurrence, among the 92 accessions, of several sets of genetically related cultivars that were differing for resistances. In a similar way, a preliminary experiment on DH lines tends to confirm the linkage between a marker and the blue-mold resistance factor. The efficiency of this process is noteworthy when compared to the high number of RAPD primers that had to be screened to reveal associations with the same resistance factors. For example, 700 decamers were screened

to find markers linked with the black root rot resistance (Bai et al. 1995). This process is actually renewed with another set of cultivars. AFLP markers associated with resistances obtained from other interspecific crosses, such as cyst nematode (*Globodera* spp.) and black shank (*Phytophthora parasitica* var. *nicotianae*), are under identification.

Assessing polymorphism on a set of diverse cultivars has been used to find associations with important phenotypic traits: seed coat color in *Brassica juncea* (Sabharwal et al. 2004), yield and yield stability in barley (Kraakman et al. 2004), biotic and abiotic stress tolerance in barley (Ivandic et al. 2003). When considering that not only disease resistances but also tobacco types or the leaf chemical composition could be related at least partially to some markers found in this study, this type of approach seems promising for *N. tabacum*. Further developments will be facilitated by association mapping methods that account for the parentage of the accessions from the reference population (Thornsberry et al. 2001).

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