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A mitochondrial molecular marker of resistance to Bayoud disease in date palm

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Abstract We have previously shown that two circular plasmid-like DNAs (the S and the R plasmids) can be found in the mitochondria of date palm (*Phoenix dactylifera* L.), a dioecious monocotyledonous tree. The two plasmids differ essentially by the absence, in the R plasmid, of a 109-bp DNA segment. Using 36 date palm varieties and employing a PCR-based approach, we show that the simultaneous presence of the R plasmid and absence of the S plasmid can be considered as a reliable molecular marker of resistance to a vascular wilt (Bayoud disease) caused by the fungus *Fusarium oxysporum* f. sp. *albedinis*. Conversely, the simultaneous presence of the S plasmid and absence of the R plasmid is correlated to Bayoud disease susceptibility The availibility of this diagnostic tool for plasmid characterization should subsequently allow simple, rapid and efficient selection of Bayoud-resistant individuals from the large number of date palms obtained by natural crosses which display good date quality.

Keywords Plants · Date palm · Bayoud disease · Plasmid-like mitochondrial DNA · DNA-based marker

Introduction

Exploitation of the date palm tree, a perennial allogamous plant, has been carried out for a long time in North African countries where this species plays an extensive human and socio-economic role. Unfortunately, a vascular fusariosis, named Bayoud disease, caused by a filamentous imperfect fungus (*Fusarium oxysporum* f. sp. *albedinis*), is at present progressively destroying date palm plantations in this region of the world. This disease, which was observed for the first time in Morocco more than a century ago in the Drâa valley, has been spreading continuously, especially in the eastward direction (Djerbi 1982). In fact, the majority of the date palm groves of Morocco and of the west side of Algeria are now affected. Tunisia appears to be as yet unaffected by the disease.

To overcome this critical problem, it is imperative that date palm selection programmes aimed at selecting varieties and natural populations which display both good fruit quality and resistance to Bayoud disease be rapidly developed as, in general, Bayoud-resistant date palm display poor fruit quality. Selected individuals should then be used to produce, as rapidly as possible, female adult trees exhibiting these beneficial traits in order to restore disease-resistant plantations.

As date palm is dioecious, hybridization programmes may be performed using progenitors known to be either Bayoud resistant or displaying good fruit quality. Any progeny of interest can then be propagated from offshoots, which develop at the base of the trees. However, this method presents two major hindrances: (1) it is very time-consuming, and (2) it is limited by the low number of offshoots produced by the mother-tree. This is why a research programme based on the use of early molecular markers for Bayoud resistance, fruit quality and sex determination, combined with techniques for the rapid propagation of the individuals of interest by in vitro culture, would be a more-desirable approach. In these conditions, the period of time necessary to select the individuals of interest would be minimized and the offspring production maximized, provided that there are no somaclonal variation effects on the genes involved in Bayoud resistance, fruit quality and sex determination.

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The identification of DNA markers linked to disease resistance or susceptibility was first achieved using RFLP technology. Subsequently, the use of PCR and its derivative, RAPD, was strongly expanded due to the ease, low cost and rapidity of the process. As far as Bayoud disease is concerned, a PCR approach has recently been successfully used to develop a diagnostic tool for *F. oxysporum* f. sp. *albedinis* detection (Fernandez et al. 1998).

To-date, three differently sized plasmid-like circular DNA structures have been isolated from the mitochondria of date palm (U plasmid: 1,160 bp, S plasmid: 1,454 bp and R plasmid: 1,345 bp) (Benslimane et al. 1994, 1996). The S plasmid differs essentially from the R plasmid by the absence, in the latter, of a 109-bp DNA segment. Since, until the present work was undertaken, only the S plasmid or the R plasmid could be found in a given variety, this structural difference enabled an easy and fast characterization of the presence of either the S plasmid or the R plasmid using a PCR approach. Preliminary Southern analyses, performed using a small number of date palm varieties, suggested that a relationship could exist between the specific presence of the S plasmid and Bayoud susceptibility, and between the specific presence of the R plasmid and Bayoud resistance.

In this paper, our investigations were extended to 36 date palm varieties originating from Morocco and Tunisia which were already characterized with respect to their resistance/susceptibility to Bayoud disease. The use of PCR technology has allowed us to propose that the presence of either the S or the R plasmid could be considered as an early molecular marker of susceptibility or resistance to Bayoud disease.

Material and methods

Plant material

Thirty six date palm (*Phoenix dactylifera* L.) varieties were used as starting material. In addition, experiments were carried out from plantlets regenerated from in vitro culture of four of these 36 varieties (Bou-Sthammi noire, Tadment, Bou-Sthammi blanche and Bou-Feggous).

DNA preparation

Total cellular DNA was prepared from leaves of either trees or regenerated plantlets according to either Aïtchitt et al. (1993) or Ouenzar et al. (1998). *Hin*dIII – cloned R and S mitochondrial plasmid DNA was isolated according to Birnboim and Doly (1979).

PCR amplification

Twenty nanogram aliquots of each total cellular DNA preparation or 2 ng aliquots of *Hin*dIII – cloned R and S plasmids were mixed, in a total volume of 25 µl, with 12.5 pmol of each oligonucleotide primer, 0.5 units of *Taq* polymerase (Appligène) and subjected to 30 cycles (94°C, 1 min; 60°C, 1 min; 72°C, 1 min) of amplification with 0.1 mM each of dATP, dCTP, dGTP and dTTP according to the manufacturer's conditions. A 2-min final extension was systematically performed. PCR amplifications were carried out in a "Crocodile II" thermocycler (Appligène). Aliquots of 10 µl from each reaction were then loaded onto a 1.4% agarose gel, separated by electrophoresis, stained with Ethidium bromide and photographed under UV light. Nucleotide sequences of the oligonucleotide primers were as follows: oli1, $^{5'}$CCT TAT ACC AGT CGT GCT T$^{3'}$; oli2, $^{5'}$AAG GCA GAT ATA ATC GGA$^{3'}$ (Fig. 1, panel A).

Molecular cloning and sequencing

PCR amplification products were excised from the gels, eluted and cloned in the pCR2.1-TOPO vector (Invitrogen) according to the manufacturer's recommendations. Nucleotide sequences were determined for both strands using an Applied Biosystem automated DNA sequencer.

Results

PCR amplification as a convenient tool for the easy and rapid identification of two related plasmid-like DNAs

Previous investigations of the mitochondrial DNA of date palm plantlets regenerated from in vitro culture of meristematic structures (Benslimane et al. 1994, 1996) had shown the presence of two plasmid-like circular structures, the S plasmid (1,454 bp) and the R plasmid (1,345 bp) essentially differing by the specific presence, in the S plasmid, of a 109-bp sequence at nucleotide positions 1,145–1,253 (Fig. 1, panel A). It is likely that an intramolecular recombination event at the two copies of an octanucleotide motif of the S plasmid (TTACAAGT, at nt positions 1,137–1,144 and 1,246–1,253) generates the R plasmid and a 109-bp DNA molecule. Apart from this 109-bp sequence, the two plasmids share 98.8% sequence similarity. Indeed, sequence comparison reveals 17 point-mutation events (Benslimane et al. 1996), nine of them localized between the deletion point in the R plasmid and the primer oli2 (Fig. 1, panel A). This suggests that if the S plasmid gave rise to the R plasmid then this probably was a historical event. It must be kept in mind that nucleotide sequences of the S and R plasmids were obtained from two date palm varieties, one of them containing only the S plasmid and the other only the R plasmid. In these conditions, sequence differences probably reflect independent evolutionary events since the recombination took place. It was thus possible to individually detect the two plasmid structures by using a pair of oligonucleotide primers to amplify, by PCR, a region spanning the 109-bp DNA sequence. Thus, two oligonucleotide primers (oli 1 and oli 2) hybridizing to sequences located upstream (at nucleotide positions 1,008 to 1,026) of and downstream (at nucleotide positions 1,363 to 1,380 in the S plasmid) from the 109-bp sequence were used to amplify either a 373-bp fragment, in the case of the S plasmid, or a 265-bp fragment, in the case of the R plasmid (Fig. 1, panels A and B). This apparent 1-bp discrepancy between the difference of the amplified fragment sizes (108 bp) and the size of the deleted fragment from the R plasmid (109 bp) is explained

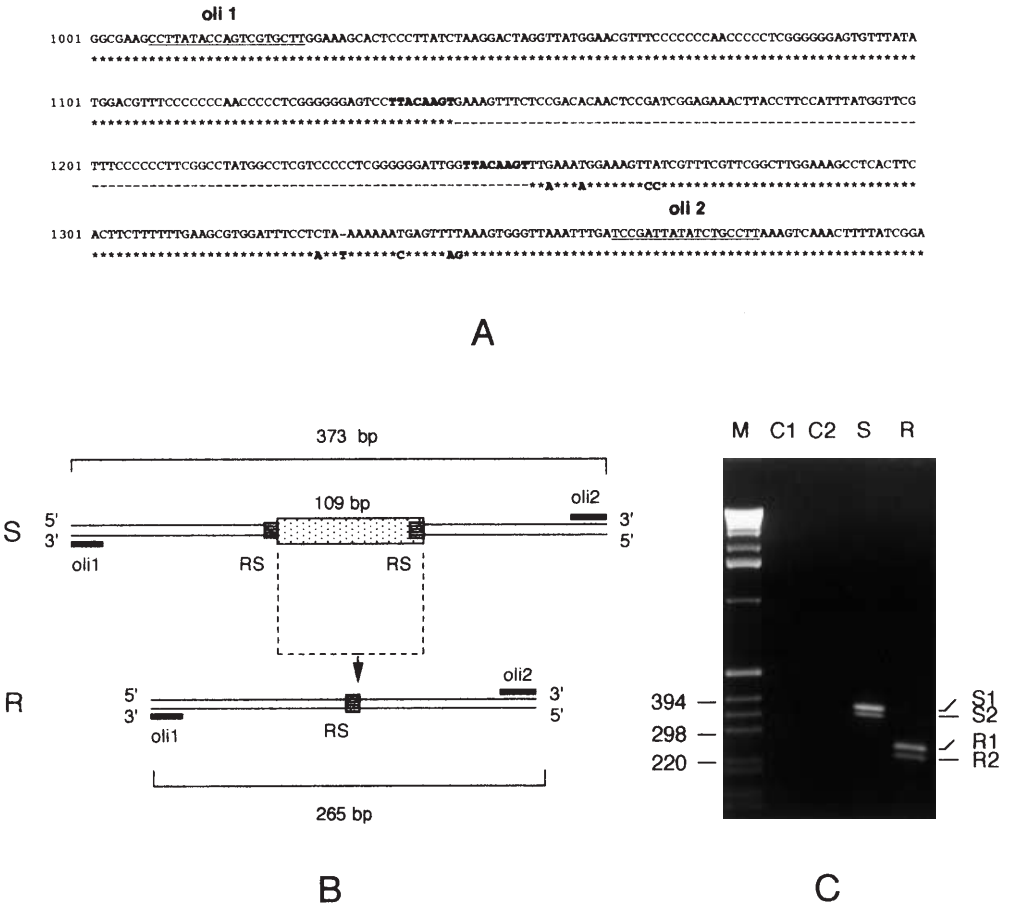


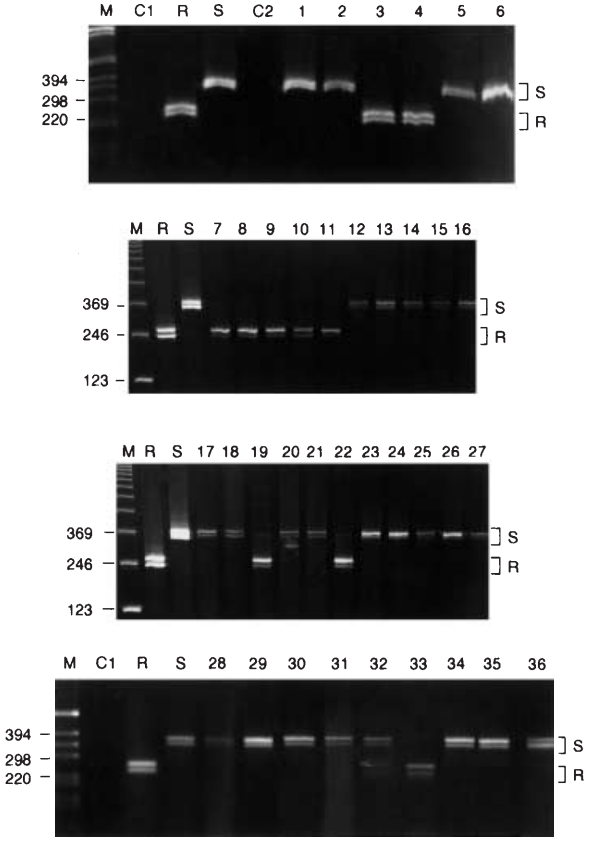
Fig. 1A–C Design of a pair of oligonucleotide primers for PCR amplification of a region of interest in the S and R plasmids. Panel **A**: alignment of the nucleotide sequence of a region of the S and R plasmids showing (1) the localization of the sequences (*underlined*) complementary to the oligonucleotide primers oli1 and oli2 used for PCR amplification, and (2) the localization of the direct repeats TTACAAGT (*bold-faced*) at the origin of the recombination event generating the R plasmid (at positions 1,137–1,144 and 1,246–1,253). Sequence identities are denoted by *stars*, and gaps (at positions 1,331 for the S plasmid and 1,155–1,263 for the R plasmid) by *hyphens*. Sequence numbering is that previously used by Benslimane et al. (1996). Panel **B**: schematic representation of the region of the S and R plasmids delimited by sequences complementary to oligonucleotide primers oli1 and oli2 (*blackened bars*), showing that a 373-bp fragment and a 265-bp fragment are amplified from the S and the R plasmids respectively. The *hatched boxes*, denoted RS, correspond to the 8-bp-long recombinogenic repeats, whereas the 109-bp *stippled box* delimits the region of the S plasmid which is not found in the R plasmid. Panel **C**: Ethidium bromide staining of a 1.2% agarose gel containing the 373-bp (*S1*) and 265-bp (*R1*) DNA fragments amplified, by PCR, from the *Hin*dIII – cloned S and R plasmids respectively. *S2* and *R2* denote accompanying amplified fragments which are systematically detected. *C1*: control including *Taq* polymerase and primers but excluding plasmid DNA. *C2*: control including S and R plasmid DNA but excluding *Taq* polymerase and primers. M: "1-kb ladder" (BRL) size standard. Fragment sizes (in bp) spanning the amplified fragments are indicated

by a 1-bp deletion (at nt position 1,331) in the amplified region of the S plasmid (Fig. 1, panel A) external to the 109-bp fragment. As shown in Fig. 1, panel C, *Hin*dIII – cloned R and S plasmids gave the expected amplified fragments (R1 and S1 fragments respectively). However, each of them was accompanied by a an approximately 25-bp shorter amplified fragment (R2 and S2 fragments respectively). To determine the origin of these extra bands, the R1 and S1 upper bands were excised from a preparative-gel electrophoresis, in such a way that no contamination by the R2 or the S2 lower band could occur, and then eluted. A part of the eluted DNA was then re-amplified and electrophoresed. Both the upper and the lower fragments were detected whereas the corresponding non-re-amplified control samples showed the presence of only the upper band (data not shown). In addition, R1 and R2 re-amplified DNA fragments were separately sequenced and shown to be 100% homologous (data not shown). It thus appears that the presence of the extra bands R2 and S2 is a consequence of the PCR amplification process. In these conditions, the oligonucleotide pair oli1 and oli2 can be considered as being suitable for a rapid and easy identification of the R and S plasmids.

Evidence for a relationship between the presence of either the S or the R plasmid and either susceptibility or resistance of the date palm to Bayoud disease

Thirty six date palm varieties whose response to Bayoud disease was known (28 of them being Bayoud-susceptible and the remaining eight being Bayoud-resistant)

sample number	variety	origin	plant material tested	plasmid (S/R)	resistance (R) or susceptibility (S) to Bayoud disease
1	V1DP	a	regenerated plantlet	S	S
2	V2DP	a	regenerated plantlet	S	S
3	V3DP	a	regenerated plantlet	R	R
4	V4DP	a	regenerated plantlet	R	R
5	V5DP	a	regenerated plantlet	S	S
6	V6DP	a	regenerated plantlet	S	S
7	**Bou-Sthammi noire**	b,c	adult tree	R	R
		b	regenerated plantlet	R	
8	**Tadment**	b,c	adult tree	R	R
		b	regenerated plantlet	R	
9	**Bou-Sthammi blanche**	c	adult tree	R	R
		b	regenerated plantlet	R	
10	Sair Layalet	c	adult tree	R	R
11	Iklane	b	adult tree	R	R
12	Bou-Feggous-ou-Moussa	b	adult tree	S	R (I)
13	Bou-Slikhene	d	adult tree	S	S
14	Aissa-Youb	c	adult tree	S	S
15	Boud-Cerdonne	c	adult tree	S	S
16	Bout Ittob	c	adult tree	S	S
17	Bou-Temda	c	adult tree	S	S
18	Oum-N'hale	c	adult tree	S	S (II)
19	Mest-Ali	c	adult tree	R	S
20	Bou-Ijjou	d	adult tree	S	S
21	Outoukdim	c	adult tree	S	S (II)
22	Jihel	b,c	adult tree	R	S
23	Agueilid	c	adult tree	S	S
24	**Bou-Feggous**	c	adult tree	S	S
		b	regenerated plantlet	S	
25	Bou-Skri	c	adult tree	S	S
26	Aherdane	c	adult tree	S	S
27	Mejhoul	d	adult tree	S	S
28	Kentichi	g	adult tree	S	S
29	Ghondi	f	adult tree	S	S
30	Khouat-Ftimi	g	adult tree	S	S
31	Kenta	g	adult tree	S	S (III)
32	Horra	g	adult tree	S/R	S
33	Deglet-Nour	e	adult tree	R	S
34	Boufagous	f	adult tree	S	S (II)
35	Ftimi	g	adult tree	S	S
36	Besser-Hlou	g	adult tree	S	S



A B

Fig. 2A, B Correlation between Bayoud resistance/susceptibility and mitochondrial plasmid identity (R and/or S). Panel **A**: thirty six date palm varieties (*numbered 1 to 36*), for which the response to Bayoud disease had been previously determined, were tested for the type of mitochondrial plasmid DNA they contained. Leaves of either adult trees or regenerated plantlets were collected in Morocco (*a*: Domaines Royaux, Meknes, *b*: I.N.R.A., Marrakech, *c*: I.N.R.A., Zagora, *d*: I.N.R.A., Errachidia) and in Tunisia (*e*: I.N.R.A.T., Degache, *f*: I.N.R.A.T., Tozeur, *g*: I.N.R.A.T., Djerid). For each variety, both the nature of the plasmid DNA (*R, S,* see panel **B**) and the response to the pathogen are indicated. The nature of the plasmid DNA found in varieties no. 7, 8, 9 and 24 (*bold-faced*) was determined, for each variety, from leaves of both adult trees and plantlets regenerated from in vitro culture of meristematic tissue. *Bold horizontal arrows* indicate varieties for which either resistance to Bayoud disease is accompanied by the presence of the S plasmid (*I*) or susceptibility to Bayoud disease is accompanied by the presence of the R plasmid (*II*), or for which the two plasmids are present (*III*). Panel **B**: Ethidium bromide staining of 1% agarose gels containing the 373-bp (*S*) or/and the 265-bp (*R*) DNA fragment amplified by PCR from cellular DNA of the 36 varieties (*numbered 1 to 36*) referenced in panel **A**. *C1*: control including *Taq* polymerase and primers but excluding DNA. *S and R*: controls using *Hin*dIII – cloned S and R plasmids respectively. *M*: "1-kb ladder" (BRL) size standard (varieties 1 to 6 and 28 to 36) or "123-b ladder" (BRL) size standard (varieties 7 to 27). Fragment sizes (in bp) spanning the amplified fragments are indicated

were analyzed for the presence of the R and the S plasmids by PCR amplification using the oligonucleotide pair described above. It should be stressed that, in this work, the varieties which are not totally resistant to Bayoud disease have been termed "susceptible". In fact, a range of intermediate levels exist between total resistance and marked susceptibility (Saaidi 1992). In addition, the sampling used in our experiments comprises a significantly greater proportion of Bayoud-susceptible varieties. This is due to the fact that, in general, date palm trees having good fruit quality are Bayoud-susceptible. Figure 2 shows, for each variety, the geographic origin, the plant material tested, the identity of the plasmid, the resistance/susceptibility of the variety to Bayoud disease (panel A) and the PCR amplification profiles (panel B). As expected, each amplified DNA fragment (either 265- or 373-bp) was accompanied by a second band, the relative amount of which differed according the DNA sample checked. In seven out of the eight Bayoud-resistant varieties only the R plasmid was detected whereas, among the 28 Bayoud-susceptible varieties, 24 had the S plasmid only and, intriguingly, one Tunisian variety (Horra) had both plasmids.

Finally, it appears that in vitro culture does not a priori generate mitochondrial structural variation, at least as far as the region of the S and R plasmids corresponding to the 373- and 265-bp DNA fragments is concerned. Indeed, results obtained from in vitro cultured offshoots of four varieties (Bou-Sthammi noire, Tadment, Bou-Sthammi blanche and Bou-Feggous) and from corresponding regenerated plants did not reveal any change in the nature of the plasmid (data not shown).

Discussion

The aim of the present study was to provide an early molecular marker for Bayoud resistance/susceptibility. This investigation was sustained by data obtained from preliminary Southern analyses performed with six date palm (four Bayoud-susceptible and two Bayoud-resistant) varieties and showing that, according to the variety, the mitochondrial genome of the Bayoud-susceptible varieties contains a an approximately 1.45-kb plasmid-like structure (the S plasmid) and that of the Bayoud-resistant varieties an approximately 1.35-kb plasmid-like structure (the R plasmid). It should be noted that the previous report of Flamand et al. (1993) shows that mitochondrial plasmids of broad bean arise by recombination and are controlled by factors in the nuclear genome. Since sequencing data revealed that the R plasmid differed from the S plasmid essentially by the absence of a 109-bp DNA segment, the PCR approach was used to screen a collection of Bayoud-susceptible and Bayoud-resistant date palm varieties with respect to the identity of the plasmid structure (S or R) which they contained. Taken together, our results favour the occurrence of a relationship between the nature of the plasmid and Bayoud resistance-susceptibility and indicate that PCR amplification using oligonucleotides oli1 and oli2 as primers could be a suitable tool for the early and easy discrimination of resistance and sensitivity to Bayoud disease. Indeed, in this varietal sampling and when only the S or the R plasmid is detected, the relationship is verified in 31 cases out of 35 (88%) whereas the chance of randomly selecting a Bayoud-resistant variety among these 35 samples is only 23% (8/35). These features could be reasonably explained if the plant is homozygous for either the dominant or the recessive allelic forms of two linked nuclear genes, one being involved in plasmid recombination and the other in Bayoud disease resistance. Exchanges of chromosomal segments through genetic crossing-over could account for the few deviations from this correlation that were observed. It must however be kept in mind that, beside the 35 varieties where only either the S or the R plasmid could be detected, one variety (Horra) had the two plasmids. It can be inferred that, in this variety, two S plasmid populations exist in the mitochondrion, one of them generating the R plasmid by recombination at the set of short direct repeats and the other being unable to recombine.

In general, date palm varieties cultivated for their good fruit quality have not originated from controlled crosses but rather from random pollination. Such individuals exist in large numbers and are by definition genetically unique. Thus, the avaibility of a reliable, simple and rapid diagnostic tool for plasmid characterization should allow efficient selection of individuals displaying both good date quality and Bayoud resistance. These individuals could then serve as explant donors for their subsequent propagation by somatic embryogenesis, a method apparently more effective than vegetative propagation (Bhaskaran and Smith 1992; El Hadrami et al. 1995).

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