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Leaf proteins as markers useful in the genetic improvement of coconut palms

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Summary

There is a need to identify genetic markers that can assist coconut (*Cocos nucifera* L.) breeding programs. With that objective, electrophoretic patterns of leaf peroxidases, endopeptidases and coomassie blue stained proteins were analysed in four cultivars ('West African Tall', 'Rennell Tall', 'Malayan Yellow Dwarf', 'Cameroon Red Dwarf'), and in the hybrids PB121 ('Malayan Yellow Dwarf' × 'West African Tall') and PB111 ('Cameroon Red Dwarf' × 'West African Tall'). The polymorphisms detected fit the expression of two alleles of a dimeric peroxidase, two alleles of a monomeric endopeptidase, and a pair of active and null alleles of a coomassie blue stained protein. Four distinctive genotypes were identified. One for each of the tall cultivars, another for both of the dwarf cultivars, and the last for both of the hybrids. Applications of the markers in breeding programs, and in research concerning reproductive biology and phylogeny, are discussed.

Introduction

Breeding programs for the coconut palm (*Cocos nucifera* L.) generally rely on the production of interpopulation hybrids, primarily between dwarf and tall cultivars. These hybrids generally yield better than their parents due to a complementarity of yield components and the possible presence of heterosis (Harries, 1991). PB121 ('Malayan Yellow Dwarf' × 'West African Tall') and PB111 ('Cameroon Red Dwarf' × 'West African Tall') are high yielding hybrids produced at a commercial scale in Ivory Coast. They have been introduced to at least 12 countries, and PB121 is claimed to be the most widely cultivated coconut hybrid in the world (Bourdeix et al., 1992; Nuce de Lamothe & Benard, 1985).

The commercial production of PB121 and PB111 is based on the assisted pollination technique, which involves emasculation and manual pollination of the mother palms (Nuce de Lamothe & Rognon, 1973). To apply this technique, dwarf cultivars are preferred as female progenitor for their precocity, higher rate of inflorescence emission, higher number of female flowers per inflorescence, and lesser height, as compared to tall cultivars (Nuce de Lamothe & Benard, 1985). Hybrids produced by assisted pollination are selected in the nursery by either of two markers. The germination speed of the seeds has proved useful whenever it differs notably in the progenitors (Whitehead, 1965). Another marker, the petiole color, is most widely used as a selection method. Seedlings with the same petiole color as the mother palm are considered self pollinations. This marker has been used to select yellow × green, and red × green hybrids (Saint & Nuce de Lamothe, 1987), to detect pollen contamination in seed orchards (Nuce de Lamothe & Rognon, 1975), and to estimate outcrossing rates (G.R. Ashburner, personal communication).

Analyses of petiole color segregation indicate that this trait fits the expression of two diallelic, unlinked loci (Bourdeix, 1988). The proposed determinism implies that selection of hybrids by petiole color can be totally reliable only if progenitors homozygous for yellow, red or green petiole are used. Even so, it would be worthless if the progenitors are the same color, or in green × yellow, and red × yellow crosses. Thus, although practical, selection of hybrids by petiole color poses important disadvantages: 1) It limits the use of the variation found in the coconut palm; 2) it can underestimate the yield of true hybrids; and 3) it is subjective and hence prone to error.

The need has long been recognised for genetic markers that can assist in hybrid production programs, as well as in studies on breeding systems, reproductive biology, population genetics, and phylogeny of the coconut palm. For these purposes, biochemical and molecular markers have been analyzed which include polyphenols (Jay et al., 1989), proteins and isozymes (Benoit & Ghesquiere, 1984; Canto-Canché et al., 1993; White et al., 1987), and random amplified polymorphic DNA (RAPD) (Ashburner, 1997). All the groups that analyzed proteins and isozymes found that leaf extracts were easily oxidised, gave low enzyme activities and inconsistent results. The best extracts were obtained from either pollen, embryos or endosperm. This implies that even if any genetic markers were identified in these tissues they could not be used to test young plants. Hence, they would be worthless to differentiate progeny in hybrid production programs. In these cases, genetic markers are required which can be detected in vegetative tissues.

Recently, we established conditions that succesfully overcame the problems previously associated with the electrophoretic analysis of coconut leaf proteins. By this approach, polymorphic patterns of peroxidases, endopeptidases and coomassie blue stained proteins were detected in genotypes introduced to México. The objectives of the present study were (1) to investigate the genetic basis of the polymorphisms detected, and (2) to determine the possibility of using these polymorphisms to differentiate the cultivars WAT, RLT, MYD, CRD, and the hybrids PB121 and PB111.

Materials and methods

Plant material. Analyses were performed with the cultivars ‘West African Tall’ (WAT), ‘Rennell Tall’ (RLT), ‘Malayan Yellow Dwarf’ (MYD), ‘Cameroon Red Dwarf’ (CRD), and the hybrids PB121 (‘Malayan Yellow Dwarf’ × ‘West African Tall’) and PB111 (‘Cameroon Red Dwarf’ × ‘West African Tall’). These genotypes come from seeds introduced to México from the Marc Delorme Station of the Institut de Recherches pour les Huiles et Oléagineux (IRHO) in Ivory Coast, Africa, from 1977 to 1979 (Manciot, 1978; Meunier, 1982). All the seedlings were quarantined before release and, to our knowledge, no selection for true hyrids was performed in the nurseries. The plants are in the experimental station ‘Benito Juárez’ of the state of Guerrero and are 18 years old.

Protein analyses. Pinnae from the spear leaf (youngest open leaf) were harvested and transported to the laboratory with the cut end immersed in water. 0.1 g of leaf lamina was ground in a pre-chilled mortar with 70 mg of polyvinilpolypirrolidone (PVPP) and 1 ml of cold extraction buffer (0.1 M ascorbic acid, 50 mM cysteine and 5 mM thiourea, pH 5.50). The homogeneous paste was pressure filtered to clarity through filter paper and immediately stored in either liquid nitrogen or an ultrafreezer (− 80 °C). Proteins were electrophoresed in a discontinuous buffer system (Davis, 1964; Ornstein, 1964). 7.5 percent polyacrylamide was used in the separating gels and 13 mA/cm^2 constant current was applied during the run. 100 μl of extract was loaded per lane. After the electrophoresis, the protein patterns were visualized by activity staining of peroxidases (Arulsekar & Parfitt, 1986) and endopeptidases (Association of Official Seed Analysts, 1991), and by coomassie blue staining of total proteins (Dunbar, 1987). For each visualization, a different population of 21 plants was analysed.

Genetic interpretation of polymorphisms. Polymorphisms were classed into basic electrophoretic phenotypes. The variation observed was genetically interpreted, in terms of tentative alleles, based on the genetic determinisms described for a number of enzyme systems from plants (Gottlieb, 1981; Torres & Tisserat, 1980).

Differentiation of cultivars and hybrids. The genetic interpretation of the polymorphisms was considered to analyze their distribution in the populations with the aims (1) to establish a genotyping scheme that differentiates the cultivars from each other, (2) to compare the distribution of phenotypes in the populations of PB121 and PB111 with that expected from the cultivar genotyping, and (3) to establish a scheme for legitimating hybrids derived from the cultivars tested.

Results

Interpretation of electrophoretic phenotypes

The basic electrophoretic phenotypes recognised appear in Figure 1. Three phenotypes of peroxidase activity (PER) were observed. These consist of either

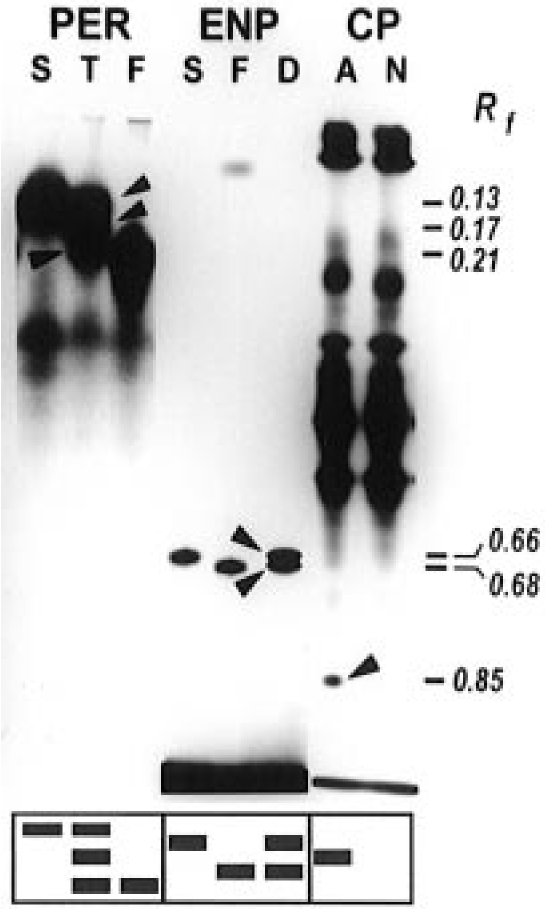

Figure 1. Electrophoretic phenotypes detected. Polymorphic bands are indicated by arrowheads, and appear schematized at the bottom. PER, peroxidases; ENP, endopeptidases; CP, coomassie blue stained proteins. S, slow band; F, fast band; D, doublet; T, triplet; A, active phenotype; N, null phenotype.

a single, fast (F) or slow (S) band, or a triplet (T) with equidistant bands. The slow and fast bands of the triplet have R_f values equal to those of the single bands in the S and F phenotypes, respectively. A single zone of endopeptidase activity (ENP) was detected that also showed three phenotypes. A slow (S) and a fast (F) band migrating very close to each other, and a doublet (D) that appeared to be composed of the S and F phenotypes. The patterns of coomassie blue stained proteins (CP) showed a fast migrating band that was present (active phenotype, A in Figure 1) or absent (null phenotype, N in Figure 1).

Phenotype variation fits the expression of a diallelic locus per system. PER and ENP allozymes would be dimeric and monomeric proteins, respectively. The simplest genetic interpretation for the phenotypes detected with CP implies a pair of active and null alleles. The subunit structure of the protein responsible for the CP A phenotype can not be determined from the variability observed.

Table 1. Number of plants per electrophoretic phenotype in each experimental population

Population	PER			ENP			CP	
	S	F	T	S	F	D	A	N
WAT	19	0	2	0	21	0	21	0
RLT	0	21	0	21	0	0	21	0
MYD	0	21	0	21	0	0	0	21
CRD	0	21	0	21	0	0	2	19
PB121	0	4	17	3	0	18	19	2
PB111	0	3	18	2	0	19	19	2

See Figure 1 for description of phenotypes.
WAT, 'West African Tall'; RLT, 'Rennell Tall'; MYD, 'Malayan Yellow Dwarf'; CRD, 'Cameroon Red Dwarf'; PB121, 'Malayan Yellow Dwarf' × 'West African Tall'; PB111, 'Cameroon Red Dwarf' × 'West African Tall'.

Analysis of cultivars and hybrids

The distribution of electrophoretic phenotypes is shown in Table 1. In ten out of the twelve cultivar/system combinations, fixed phenotypes were detected. With PER and ENP, these consisted of either of their respective S and F phenotypes, whereas with CP either the A or N phenotype was fixed. Two cultivar/system combinations showed, in addition to the above phenotypes, a second phenotype in such a low proportion as 9.5 percent. These were WAT/PER in which both S and T (minor phenotype) were detected, and CRD/CP in which both A and N (minor phenotype) appeared.

With each of the systems, PB121 and PB111 showed combinations of a major and a minor (lesser than 20 percent) phenotype. The combinations of major/minor phenotypes were, for PER, T/F; for ENP, D/S; and for CP, A/N.

Discussion

The loci detected with PER, ENP and CP will be referred to as *Per1*, *Enp1* and *CP1*; the slow and fast allozymes of both *Per1* and *Enp1* will be named S and F; A and N will be the denominations used for the active and null alleles of *CP1*. According to these denom-

Table 2. Genetic interpretation of the electrophoretic phenotypes

System/locus	Electrophoretic phenotype	Genotype	Apparent subunit structure
PER/*Per1*	S	SS	Dimer
	F	FF	
	T	SF	
ENP/*Enp1*	S	SS	Monomer
	F	FF	
	D	SF	
CP/*CP1*	A	AA or AN	Uncertain
	N	NN	

See Figure 1 for description of phenotypes.

Table 3. Genotypes assignable to the studied cultivars

Cultivar	Loci		
	Per1	*Enp1*	*Cp1*
WAT	SS	FF	AA
RLT	FF	SS	AA
MYD	FF	SS	NN
CRD	FF	SS	NN

WAT, 'West African Tall'; RLT, 'Rennell Tall'; MYD, 'Malayan Yellow Dwarf'; CRD, 'Cameroon Red Dwarf'.

inations, Table 2 presents the equivalences between phenotypes and genotypes.

Considering the genetic equivalences of Table 2, the phenotype distributions shown in Table 1 suggest that each of the cultivars is homozygous in at least two of the loci. WAT would be homozygous for *Enp1*-F and *CP1*-A; RLT for *Per1*-F, *Enp1*-S and *CP1*-A; MYD for *Per1*-F, *Enp1*-S and *CP1*-N; and CRD for *Per1*-F and *Enp1*-S. The *Per1* heterozygotes detected in WAT could be due to cross-pollination of the mother palm, as this cultivar is known to be allogamous (Rognon, 1976). It is also possible that *Per1*-F be a normal component of the genetic structure of the population analyzed. The presence of the CP A phenotype in CRD suggests that *CP1*-A is a normal component of its genetic structure, as this cultivar is autogamous (Rognon, 1976). In such a case, the highest frequency of this allele would be 0.095, corresponding to the event that the two palms with A phenotype are homozygotes. In WAT, the allele frequency observed for *Per1*-F is 0.024.

Such low allele frequencies are susceptible to become zero in successive generations by gene drift effects. However, for the alleles to be reliably applied in the differentiation of the studied cultivars, pure genotypes should be selected by removing the palms with minor alleles. Should this be done, the classification scheme of Table 3 would be applicable. It shows three combinations of homozygous genotypes; one of them corresponds to the dwarf cultivars, and the others correspond one to each of the tall cultivars.

Assuming that the genotyping scheme of Table 3 is applicable, the populations of PB121 and PB111 showed both the expected phenotypes (PER T, ENP D, and CP A) and those which would correspond to their respective dwarf progenitor. Depending on the system, the percentages of plants with the expected, hybrid phenotypes, appeared in 81 to 90 percent of PB121, and in 86 to 90 percent of PB111. The differences would be a consequence of the sample size used, as different populations were analysed with each system.

The populations of hybrids analysed in this study come from non-selected progenies of crosses performed by IRHO's program for hybrid seed production. By using the petiole color markers, Sangare & Rognon (1980) estimated the pollination efficiency of this program to range from 93 to 97 percent. This estimate was made with the progenies of crosses involving yellow and red dwarfs as female progenitors during the nursery stage. The higher than expected percentages of plants with dwarf phenotypes could have occurred as analyses were performed with adult plants, after possible contaminations or unintended selections during transport, quarantine, germination and final planting in the field.

We report for the first time the possibility to get reproducible results by the electrophoretic analysis of native proteins from coconut leaves. By this approach, genetic markers were identified that can be used in breeding programs, as well as in basic studies on population genetics and reproductive biology. Applications of the markers include:

1) Cultivar differentiation. The classification scheme of Table 3 would be applicable if populations that are homozygous in *Per1*, *Enp1* and *CP1*, are selected. Such a scheme does not differentiate MYD and CRD of each other, for which appropriate markers remain to be identified.
2) Progeny legitimacy. Assuming that the classification scheme of Table 3 is applicable, 8 out of the 12 performable crosses would give progenies with phenotypes different to those of the mother progenitor. Progenies of RLT × dwarf and dwarf × dwarf crosses would have phenotypes identical to those of the mother palms.

3) Estimation of pollen contamination. Distances between seed orchards and neighboring plantations that prevent pollen contamination depend on the environmental conditions and the type of pollinators occurring in the region (Ashburner, 1995; Free et al., 1975; Hedström, 1986). Such distances need to be experimentally determined, and the protein markers would be of great value in estimating pollen contamination when the 8 cultivar combinations above mentioned are involved.
4) Studies on breeding systems. Four breeding systems have been identified in the coconut palm which range from strict allogamy, as in WAT, to strict autogamy as in MYD (Rognon, 1976). Outbreeding rates have been estimated only by the analysis of petiole color in some yellow populations at New Guinea (G.R. Ashburner, personal communication). The protein markers could be of aid in performing this type of study.

At the moment, literature on use of DNA markers in coconut palm research is limited to a study of genetic diversity by RAPD markers (Ashburner et al., 1997). For this purpose, RAPD will prove a more powerful approach than isozyme analysis. Indeed, isozyme analysis relies on diverse and sometimes cumbersome techniques of visualization, which demand a great number of chemicals. In contrast, new sets of RAPD markers can be generated by simply changing a single reagent, an oligonucleotide, in a basic protocol. The first reports on use of RAPD markers in the applications discussed above might be expected to appear in the near future. It should be noticed, however, that RAPD is a high demanding technique in terms of equipment, and for the training and skills required to get reproducible results. For some routine applications of marker assisted selection, as is the case for cultivar differentiation and progeny legitimacy, coconut palm breeders might find analysis of leaf proteins easier to perform. The protein markers described here, with any other genetic markers of coconut palms that may be described in the future, will broaden the alternatives available to breeders of this perennial crop.

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