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Inheritance and genetic mapping of self-incompatibility in *Coffea canephora* Pierre

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Abstract Cross-compatibility behaviour of doubled haploid (DH) and hybrid genotypes of *Coffea canephora* was established using both phenotypic bioassay and in situ seed-set examination. The availability of DHs provided the opportunity of working with genetically homogenous pollen and female parents. The aniline blue fluorescence (ABF) method was applied to detect callose accumulation in pollen and pistil. Clear cross-compatibility/incompatibility situations were observed and confirmed by in situ seed-set analysis. Cross-compatibility analysis of hybrid combinations involving different DHs corroborated the crossing behaviour observed at the DH level. Expression of the self-incompatibility system did not appear to be affected by the low vigour of the DH. The crossing-behaviour distribution observed within DHs derived from clone IF200 confirmed that self-incompatibility in *C. canephora* is a gametophytic self-incompatibility system controlled by a single locus (S-locus). Reduced seed-set developments following incompatible crosses may indicate the occurrence of pseudo-incompatibility. Molecular marker linkage analysis showed that the S-locus is associated with an RFLP marker on linkage group 9. The availability of a linked DNA marker should facilitate the genetic analysis of self-incompatibility in relation to coffee breeding programmes.

Key words *Coffea canephora* · Self-incompatibility · Gametophytic system · S-locus · RFLP

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Introduction

Self incompatibility (which will be referred to as SI) in flowering plants is a genetically controlled mechanism of sexual reproduction that prevents inbreeding and promotes outcrossing (de Nettancourt 1977). At least two distinct systems exist, the sporophytic SI system and the gametophytic SI system and both are widespread throughout the angiosperms (Charlesworth 1985). The term gametophytic refers to the fact that the SI phenotype of the pollen is determined by its own haploid genotype. Gametophytic SI appears to be controlled by a single locus (the S-locus) in a large number of species involving different families, such as the Solanaceae, the Rosaceae and the Onagraceae (Newbigin et al. 1993), but there are more complex systems. For example, SI is determined by two loci in some grasses (Hayman and Richter 1992), while four loci control SI in sugarbeet, *Beta vulgaris* (Larsen 1977), and *Ranunculus acris* (Osterbye 1975).

Coffee-trees belong to the tribe *Coffeae* in the family Rubiaceae (Bridson and Verdcourt 1988). At present approximately 100 taxa have been identified in the subgenus *Coffea* (Charrier and Berthaud 1985), although, commercial coffee production relies on only 2 species, *Coffea arabica* and *C. canephora*. All *Coffea* species are considered to be self-incompatible except for the unique tetraploid species *C. arabica* and 2 diploid taxa, *C. sp.* Moloundou and *C. sp.* X, which have been observed to be self-fertile under self-pollination conditions. The presence of a gametophytic SI system in the diploid species *C. canephora* Pierre was first suggested by Devreux et al. (1959) and by Conagin and Mendes (1961). Based on observations of pollen-tube growth in the style as visualised by the lactophenol-cotton blue method (Thompson 1979), following crosses between plants of different degrees of relatedness, Berthaud (1980, 1986) established that SI in *C. canephora* is gametophytic and apparently controlled by a single locus with multiple alleles. Accurate measurements were

difficult since both the stylar cells and the pollen tubes are stained deep blue by this method and heterozygous pollen parent genotypes were used.

The purpose of the study presented here was to confirm the genetic control of self-incompatibility in *C. canephora* and to identify S-allele genotypes in order to map self-incompatibility loci for further genetic study. The cross-compatibility behaviour of doubled haploid (DH) and hybrid genotypes has been established using both phenotypic bioassay and in situ seed set examination. DHs resulting from haploid plant production followed by chromosome doubling (Couturon 1982) provided the opportunity to study completely homozygous genotypes (Lashermes et al. 1994a). The aniline blue fluorescence (ABF) method (Linskens and Esser 1957; Dumas and Knox 1983) was applied to detect callose accumulation in pollen and pistil, in association with in vitro pollination. We have used a recently constructed coffee genetic map (Paillard et al. 1996) to identify molecular markers that are linked to the gene controlling SI.

Materials and methods

Plant material

Twenty-three DHs produced from clone IF200 and nine hybrids between DHs derived from IF200 and a DH produced from clone IF160 (DH 160-02) were used. The DHs were produced according to the method of Couturon (1982) which is based on haploid plants occurring spontaneously in association with polyembryony. Hybrid genotypes were obtained using standard hybridisation techniques. All plants were grown in the field at the coffee breeding station, Man, Ivory Coast.

In the gametophytic SI system, an incompatible mating occurs when the S-alleles carried by the haploid pollen matches either one of the S-alleles present in the diploid style. In contrast, successful (i.e. compatible) matings occur when the S-allele carried by the haploid pollen is different from either of the alleles carried by the diploid style. If SI in *C. canephora* Pierre is governed by a single locus, the clone IF200 would be heterozygous (S_xS_y), and the DHs derived from IF200 would exhibit two cross-compatibility phenotypes corresponding to two genotypes (S_xS_x, S_yS_y) in a ratio 1:1, as shown in Table 1. Furthermore, the hybrids between a constant DH parent (DH 160-02) and different DHs derived from clone IF200 are expected to present a cross-behaviour identical to that exhibited by the DH derived from IF200 when crossed with homozygotes S_xS_x as well as S_yS_y.

ABF method

Buds were harvested the day before flowering. After emasculation, pistils from the different genotypes were collected and preserved in humid boxes to prevent the stigmas from drying up (Berthaud 1980). Buds of four DHs derived from IF200 (200-16, 200-30, 200-106, 200-293) were stored for 1–2 days before collection of pollen from dehiscing anthers. Previous cross-pollination testing indicated that DH 200-16 and DH 200-30 as well as DH 200-106 and DH 200-293 are cross-compatible genotypes. After 48 h the pistils were pollinated using a small brush to apply pollen of different origins. Twenty-four hours after pollination, the pistils were placed in small glass bottles and fixed with a solution of FAA made up of 10% formaldehyde (at 40%), 10% glacial acetic acid and 80% ethanol (at 95%). The samples were thereafter kept in a refrigerator at 4 °C pending observation.

Prior to microscopic examination, the pistils were treated with 1 *N* NaOH for 24 h and washed with distilled water (five changes, three times 5 min, 1 h and 5 min). Pistils were stained for 12 h with 1% aniline blue prepared in 0.1 *M* K_2PO_4 (Martin 1959). Finally, the pistils were placed on slides and, after crushing, were examined by fluorescence microscopy. Usually, five pistils per cross were examined, and the penetration level of the pollen tubes into each pistil was ascertained.

Seed-set trial

Cross-behaviour of the hybrid genotypes was ascertained in situ by manual pollination. For each genotype, four branches of about 300 flowers were selected. After emasculation, cross-pollination was achieved with pollen of either DH 200-16, DH 200-30, DH 200-106 or DH 200-293 according to standard hybridisation techniques. The fruit set was estimated for each cross (branch) as the percentage of flowers which set fruit 9 months later. For each cross, a seed germination test was performed in sawdust boxes on a sample of a minimum of 30 seeds.

Molecular marker linkage analysis

A genetic map using restriction fragment length polymorphisms (RFLP) and random amplified polymorphic DNA(RAPD) was constructed (Paillard et al. 1996) based on a DH population of 85 individuals derived from IF200 that included the 23 DHs analysed in this study for cross-compatibility. The search for any association between molecular markers and cross-behaviour was accomplished by two-point linkage analyses using the MAPMAKER version 3.0 computer package (Lander et al. 1987). A minimum LOD score of 4 was chosen.

Results

The cross-compatibility of 23 DHs derived from clone IF200, when hybridised with either DH 200-16 or DH 200-30, was determined using the ABF method. Two situations were discerned (Fig. 1):(1) the presence of pistils in which the pollen tubes had clearly reached the basal region of the style; (2) all of the pistils showed exclusively pollen tubes arrested either in the internal layer of the sigma or in the adjacent part of the style. On

Table 1 Expected genotypes of plants (DHs or hybrids) derived from clone IF200 in a gametophytic self-incompatibility system controlled by a single locus (S) and their crossing behaviour when pollinated with different pollen parent genotypes

Plant material		DHs derived from IF200 (S_xS_y)		Hybrids between DH 160-02 (S_zS_z) and DHs derived from IF200	
Genotype (frequency)		S_xS_x(50%)	S_yS_y(50%)	S_zS_x(50%)	S_zS_y(50%)
Crossing behaviour	Pollen parent S_xS_x	−	+	−	+
	Pollen parent S_yS_y	+	−	+	−

+, Denotes compatible cross; −, denotes incompatible cross

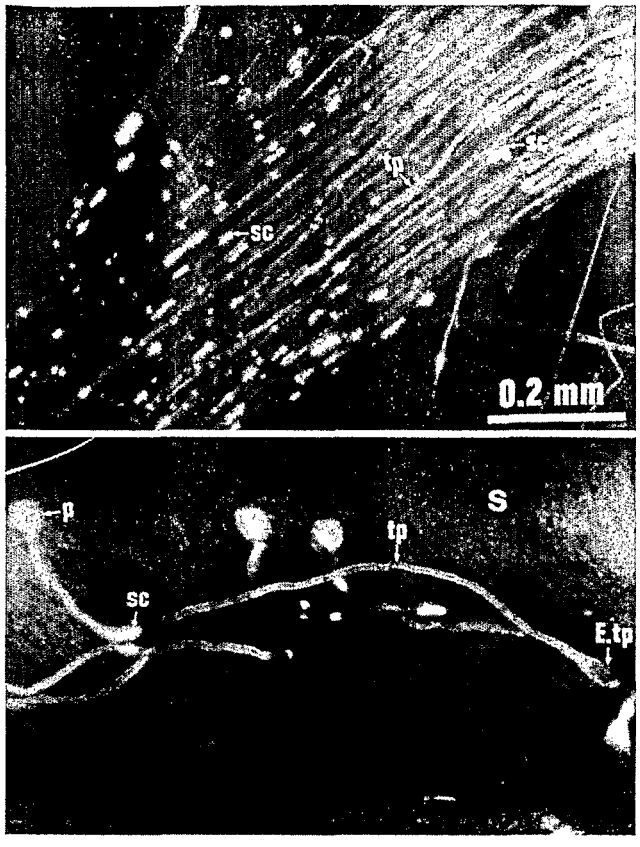

Fig. 1 Fluorescence micrographs (ABF method) showing pollen-tube growth within the style after a compatible (*top*) and an incompatible (*bottom*) pollination. The pollen tube (*tp*) walls fluoresce, and at regular intervals there are deposits of intensively fluorescent material known as callose plugs (*sc*). In the incompatible cross, growth of the pollen (*p*) tube is arrested (*E.tp*), usually in the internal layer of the stigma (*S*) or the adjacent part of the style

the basis of these observations, cross-compatibility or cross-incompatibility was assigned for each mating (Table 2). Self-incompatibility of both DH 200-16 and DH 200-30 was confirmed. DH appeared to be compatible with exclusively one pollen parent, either DH 200-16 or DH 200-30. According to their cross-behaviour, the DHs were distributed in two crossing types (Table 2). The observed distribution (11/12) did not differ significantly from a 1:1 ratio ($\chi^2 = 0.04$; $P = 0.8$) and conformed to that expected, as described above. Similar results were obtained using pollen of DH 200-106 and DH 200-293 (data not shown).

Seed sets of nine hybrid genotypes between a constant DH parent (DH 160-02) and different DHs derived from clone IF200 were observed after cross-pollination with two distinct pollen parent genotypes (Table 3). The hybrids showed a drastic reduction in fruit set when pollinated by a DH derived from IF200 belonging to the same crossing-type group as the DH involved in the formation of the hybrid. On the other hand, a fruit set was observed when the DH used as pollen parent had a different crossing type. Furthermore, in the case of intra-crossing-type group mating, the few seeds which

Table 2 Cross behaviour determination for 23 DHs derived from IF200 based on callose response observation following in vitro pollination with different pollen parent genotypes

Genotype	Callose response[a]		Deduced crossing type[b]
	Pollen parent		
	DH 200-16	DH 200-30	
DH 200-03	−	+	1
DH 200-05	−	+	1
DH 200-15	+	−	2
DH 200-16	−	+	1
DH 200-19	+	−	2
DH 200-20	+	−	2
DH 200-22	−	+	1
DH 200-25	+	−	2
DH 200-30	+	−	2
DH 200-45	−	+	1
DH 200-72	+	−	2
DH 200-73	−	+	1
DH 200-106	−	+	1
DH 200-128	−	+	1
DH 200-168	−	+	1
DH 200-181	+	−	2
DH 200-184	+	−	2
DH 200-198	−	+	1
DH 200-228	+	−	2
DH 200-246	+	−	2
DH 200-248	+	−	2
DH 200-257	−	+	1
DH 200-293	+	−	2

[a] +, compatible; −, incompatible
[b] Types 1 and 2 were arbitrarily assigned to genotypes cross-compatible with DH 200-30 or DH 200-16, respectively

were produced showed a reduced germination rate (Table 3). Average germination rates of the seeds obtained following either intra- or inter-crossing-type group mating were 16% and 75%, respectively. The presence of an embryo was verified for the seeds resulting from intra-crossing-type group mating (data not shown). Although seeds without an embryo were observed, most seeds had an embryo.

Of the 147 molecular markers (47 RFLP and 100 RAPD) tested for their association with cross-compatibility, 3, 1 RFLP (g1069D) and 2 RAPD (OO20 and OM15), appeared to be linked to the crossing-type at an LOD threshold of 4. These 3 markers constituted linkage group 9 of the coffee genetic map of Paillard et al. (1996). The estimated location of the locus (S) controlling cross-compatibility on the linkage group 9 is presented in Fig. 2. No recombinant was detected between g1069D and the S-locus in the population of 23 DHs. The upper confidence limit of the recombination frequency was calculated using the binomial distribution to be 12% and 18% at the 0.05 and 0.01 probability levels, respectively.

Discussion

An original aspect of this study was the use of DH plant material. The possibility of dealing with genetically

Table 3 Fruit set and seed germination rate of hybrid genotypes involving different DHs derived from IF200 and a constant DH derived from IF160 (DH 160-02) after cross-pollination with pollen parent types 1 and 2

[a] Crossing type of DH derived from IF200 as deduced from callose response observation is indicated in parentheses
[b] Types 1 and 2 consisted of DH 200-16, DH 200-106 and DH 200-30, DH 200-293, respectively

Genotype[a]	Pollen parent[b]			
	Type 1		Type 2	
	Fruit set (%)	Seed germination rate (%)	Fruit set (%)	Seed germination rate (%)
DH 160-02 × DH 200-03 (1)	3	5	48	46
DH 160-02 × DH 200-05 (1)	3	40	44	55
DH 160-02 × DH 200-15 (2)	61	71	3	0
DH 160-02 × DH 200-16 (1)	2	31	61	79
DH 160-02 × DH 200-19 (2)	74	71	1	-
DH 160-02 × DH 200-20 (2)	32	79	12	1
DH 160-02 × DH 200-22 (1)	5	0	55	83
DH 160-02 × DH 200-25 (2)	48	91	3	47
DH 160-02 × DH 200-30 (2)	64	96	4	0

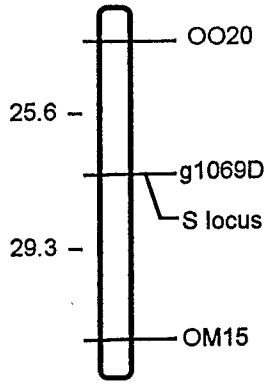

Fig. 2 Map of linkage group 9 of *Coffea canephora* (Paillard et al. 1996) showing the estimated location of the self-incompatibility gene (S-locus). Distances are given in centiMorgans (cM) (Kosambi function)

homogeneous pollen and female parents led to clear cross-compatibility/incompatibility figures and simplified the genetic analysis. DH genotypes of *C. canephora* showed low vigour, the result of a strong, negative effect of homozygosity (Lashermes et al. 1994a). However, unlike some agronomic characteristics (Lashermes et al. 1994b), expression of the SI system did not appear to be affected, and analysis of hybrid combinations involving different DH corroborated the crossing behaviour observed at the DH level. Distribution of the crossing behaviour observed within the DHs derived from the clone IF200 conforms to that expected with a cross-compatibility controlled by a single locus. The presence in *C. canephora* of a gametophytic SI system determined by a single locus (Berthaud 1980) is therefore confirmed.

The presence of a reduced seed set on the hybrid genotypes after crossing with an incompatible pollen parent suggests that SI in *C. canephora* is surmountable to a certain extent. Ostensibly, pollen grains which share an S-allele with the pistil may nevertheless be functional. This phenomenon is referred to as pseduo-incompatibility and has been demonstrated in numerous species (Ascher 1976; de Nettancourt 1977; Levin 1985). However, in the present study, the low germination rate of the seeds produced and the presence of embryo-less seeds suggest that these seeds most likely resulted from parthenogenesis. Similarly, the possibility that the seeds arise from pollen contamination is remote. However, a definitive interpretation of this phenomenon requires further investigation. In particular, the biparental origin of the seed produced could be checked using molecular markers.

The callose response as observed by the ABF method provides a useful phenotypic bioassay by which to study the incompatibility system. In most species with gametophytic self-incompatibility, pollen-tube growth is arrested in the style or ovary (de Nettancourt 1977). However, in *C. canephora*, the site may be within the stigma as reported in Graminae, Onagraceae and *Phlox* (Levin 1985). Cross-compatibility/incompatibility situations deduced from the ABF observations were confirmed by in situ seed-set analysis. Therefore, cross-behaviour determination of a large number of crosses could be achieved without seed-set trials, which are time-consuming and costly. In addition, the use of seed set to determine whether plants share S-alleles is less desirable than the observation of pollen behaviour on stigmas because failure to set seed might not result from pollen-pistil incompatibility. The ABF examination as performed in this study can be strongly recommended for further investigation.

The S-locus controlling cross-compatibility appears to be associated with an RFLP marker, g1069, localised on linkage group 9 (Paillard et al. 1996). This constitutes the first gene mapped on the coffee genome. This linkage was established on a size-limited DH population and should be assessed on a larger number of DHs. However, the availability of such an RFLP marker is very useful. A marker linked to the S-locus enables the tagging of SI in different *Coffea* species and the study of relationships with self-fertility, and should lead to a better understanding of the genetic base and evolution of SI in the genus *Coffea*. For instance, tightly linked RFLPs may be used in a marker-based selection programme to facilitate the introgression of the self-fertility character of a wild taxon such as *C. sp.* Moloundou to the cultivated species *C. canephora* (Louarn 1992).

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