The genetic and physiological basis of incompatibility in cocoa

Introduction

There is an increasing need for cocoa breeders to produce new varieties that can produce high yields of good quality cocoa even under high disease and pest pressures. In the production of such new varieties breeders have to give due consideration to the complex pollination incompatibility system that exists in cocoa. Many of the traditionally grown varieties such as Amelonado and Comum are self-compatible and so can produce seed by self-pollination or by pollination from neighbouring trees, which are usually closely related and often originate from the same pod. However, many cocoa genotypes are self-incompatible and usually produce pods only following pollination by a cross-compatible (usually unrelated) clone. Such trees include most of the Upper Amazon types that feature prominently in breeding programmes as a source of disease resistance. The cross-compatibility status of self-incompatible clones is controlled by a single genetic locus with a large number of alleles and is determined by the genotype of the parents rather than by that of the pollen grain itself. There are consequently a large number of possible cross-compatibility groupings. At present, the cross-compatibility status of any pair of genotypes is determined empirically by hand pollination. The incompatibility reaction of cocoa, like other crops, is subject to environmental influences and will break down under certain (only partly characterised) conditions. It follows that the results of a single crossing event cannot be taken as a reliable indicator of the compatibility grouping of a particular clone. Attempts to provide information on compatibility groupings of a wide range of clones that could be used in a database, such as the ICGD, would require large numbers of crosses over a number of years and/or in different locations. This approach would be impractical for significant collections of cocoa germplasm.

Nevertheless, information of this type is of great significance to the breeder for a variety of applications. These include:

1. To identify parents that are reliably self-incompatible to ensure that all the seed produced are F_1 hybrids
2. To establish fully inter-compatible varietal mixes for planting to ensure the maintenance of on-farm biodiversity in a sustainable farming system.
3. To identify clones for the generation of homozygous breeding lines

The ability to manipulate the incompatibility system to convert normally self-incompatible clones into self-compatible ones would also have practical benefits:

1. To increase the homozygosity of a self-incompatible elite clones so that seed progeny can be propagated rather than cuttings/buddings.
2. To produce homozygous breeding lines from self-compatible clones. Once produced, these plants could then be allowed to revert to self-incompatibility and used as parents to produce F1 hybrid seed.
3. Allow F2 mapping of a self-incompatible F1 population

The main aim of this project is to develop one or more simple assays to test for incompatibility grouping that are independent of environmental influences and could form an early screening procedure for use on seedling material. The test(s) can then be applied to a wide range of material held in the quarantine collection at Reading, and eventually used as part of the characterisation process at the International Cocoa Genebank, Trinidad and other collections. The data generated would make a valuable contribution to the ICGD. Identification of signalling proteins responsible for the recognition reaction may allow the development of a simple system to mask the incompatibility recognition reaction. At the same time, the anatomical and biological basis of the rejection process will be studied on a more fundamental level. In particular, the interaction between sperm cells and the recipient egg cell apparatus will be studied using confocal microscopy.

Scientific Background

Self-incompatibility in flowering plants is a biochemical process that prevents self-fertilization. The rejection process usually occurs on the stigmatic surface or else within the conducting tissue of the style. There are essentially two broad categories of self-incompatibility:

Gametophytic incompatibility: where the incompatibility reaction is determined by the gametophyte (pollen grain).

Sporophytic incompatibility: where the genotypes of the maternal and paternal parents determine the incompatibility response.

Gametophytic incompatibility is by far the more common of the two states in higher plants and is typified by bicellular pollen at dispersal, a wet stigmatic surface and an incompatibility reaction that takes place in the style. Transcribed factors (nucleic acids or proteins) originating from the pollen tube then cause a recognition reaction within the style. This is followed by an incompatibility response. Typically, this is apparent as the accumulation of a callose at the pollen tube tip, followed by bursting of the tube itself.
Sporophytic incompatibility is a much less common system and is largely restricted to the Fabaceae (Leguminosae) and the Asteraceae (Compositae). It is characterised by species possessing tricellular pollen at dispersal, dry stigmata and an incompatibility reaction that occurs on the surface of the stigma. Proteins and glycoproteins are deposited from the tapetum layer of the anther onto the surface of developing pollen grains prior to pollen release. The chemicals held in the walls of the pollen exine are released (as pollen kit) onto the dry stigmatic surface of the female shortly after pollination. Recognition reaction between these chemicals and the maternal stigmatic tissues trigger an incompatibility response on the stigmatic surface that prevents pollen germination and penetration.

The incompatibility system of cocoa is unique and differs from both of these classical systems in several ways (Knight and Rogers, 1953, 1955 and Cope, 1958,1979, 1962). First, the incompatibility reaction itself occurs after the two sperm cells have been released from the pollen tube and into the embryo sac. As a consequence, it results in abortion of the embryo sac and the failure to produce a viable zygote. This makes the system appear inefficient when compared with the more conventional systems in which incompatible pollen are eliminated away from the (valuable) embryo sacs.
Second, the incompatibility reaction results in the abscission of the entire flower. Finally, the incompatibility reaction itself is sporophytically determined although the rejection process occurs distant from the maternally contributed pollen kit. This supposition is not easily explained. Bartley and Cope (1973) suggested that proteins produced prior to meiosis could be responsible. For this to be so, however, the proteins (or mRNA) must not only be stable but must also remain active despite several levels of dilution caused by a minimum of four divisions that occur after PMC production. Furthermore, the complementary proteins must behave similarly in the megagametophyte. A far more realistic model was proposed recently by Aneja et al (1994). These authors hypothesised that self-incompatibility in *Theobroma* may be the result of two processes, one occurring during pollen germination (pollen -stigma recognition response) and the other at the stage of gametic fusion (rejection response). Several lines of evidence support this model. Aneja et al (1994) found that incompatible self-pollen of clone IMC 30 failed to penetrate the stigmatic surface. However, if these flowers were stimulated with elevated concentrations of CO2 the incompatibility reaction was overcome and seed set. Similarly, Lanaud (1987) found that mixing incompatible self-pollen with those from a compatible source (mentor pollination), the incompatibility reaction could be overcome. The model proposed by Bartley and Cope (1973) (stable protein/mRNA) model cannot explain these findings but they can be explained as a failure of the stigmatic/pollen kit recognition process (Aneja et al model). The close relatives of *Theobroma* also throw light on the evolution of the system. The genus *Cola* has a single locus, sporophytic incompatibility system in which the incompatibility reaction occurs on the stigma (Jacob, 1980). With few exceptions, all other members of the family have the same system, suggesting the recognition part of the reaction in *Theobroma* may also be stigmatic. More recently, Baker and Hasenstein (1997) have found that pollination with incompatible pollen is positively correlated with the production of ABA and ethylene. IAA levels remained stable in incompatible pollinations but rose after compatible crosses. They inferred that incompatibility reaction is a two-stage process. The recognition reaction stimulated changes in ABA levels that are required for subsequent abscission. A second reaction occurs after pollen tube reaches the ovule and stimulates IAA and ethylene production. These then invoke the incompatible response. Thus, the recognition reaction on the stigmatic surface is distinct from the two-stage rejection response mediated by these hormones.

Research needs

The rejection response by the maternal parent is unlikely to differ greatly between genotypes and will be difficult to manipulate. The recognition reaction, on the other hand, is almost certain to be caused by proteins or glycoproteins carried in the pollen kit within the exine layer of the pollen grain. Identification and characterisation of these proteins would allow rapid and robust classification of cocoa incompatibility groupings held in germplasm collections. Such data would be extremely amenable for inclusion in the ICGD and would accurately predict the cross-compatibility of cocoa clones. This ability would be independent of environmental influences (e.g. glasshouse conditions, pollination procedure, age of plant) that can affect seed set in incompatible crosses and could have

significant strategic importance in the medium to long term. There is a growing interest in more sustainable cultivation of cocoa and for the development of material suitable for cultivation on small-holdings. The production of stable mixtures of seed containing predetermined mixtures of F1 hybrids or siblings from homozygous parents would maintain on-farm biodiversity and provide protection against local climatic variables and losses from disease. The production and performance of such mixes relies on a firm knowledge of the cross-compatibility on the constituent genotypes. Additionally, the production of homozygous breeding lines in cocoa breeding programmes would allow the production of uniform seed progenies and is a requirement for the production of F1 hybrid seed varieties. A routine and reliable system to overcome self-incompatibility would greatly benefit the production of such plants. This may be achieved by the application of proteins/glycoproteins responsible for the recognition reaction resulting in 'masking' of the incompatible signal. Similar but less reliable effects are possible at present through the use of mentor pollination.

Research programme

It is proposed that support be provided for a part-time research assistant (Caroline Ford) to study the recognition processes during incompatibility in *Theobroma* cacao to lead to a higher degree by research. The programme covers five years of research which should lead to a PhD. However, funding for three years is requested in the first instance with provision for a two year extension to allow the completion of the PhD. The research will focus primarily on the genetic and biochemical basis of the initial recognition reaction (on the stigmatic surface) but will also re-examine the anatomical symptoms of the egg-sperm interaction using techniques unavailable to earlier studies. Results generated will be used to develop a simple scoring system for the characterisation of incompatibility in clones maintained in the Quarantine collection and the data generated will be incorporated into the ICGD.
A provisional strategy and timetable is outlines below. However, it should be recognised that the direction of research towards the end of such a five year project may have to change in the light of early results, the literature and also as opportunities arise from new techniques.

Strategy

The principal goal is to isolate and then characterise the proteins responsible for the recognition reaction. The secondary aim is to study the sperm-egg cell interaction.

Protein isolation from the pollen will be attempted in two ways.
First, to isolate the proteins from the pollen-kit, isolate, purify by electrophoresis and then characterise (amino acids, mRNA and DNA sequences). Incompatibility groupings will be defined on the basis of sequence or length divergence. Tests will be developed to exploit the polymorphisms. These may be protein assays based on size or conformational differences (electrophoresis). Alternatively, they may be PCR-based assays to exploit size differences or small DNA sequence differences.
Second, proteins responsible for incompatibility of several sporophytic species have already been characterised and their DNA coding sequences deduced. Primers will be designed to amplify similar sequences from cocoa DNA. Sequence differences between cross-compatible but self-incompatible clones will be used to identify alleles responsible for the incompatibility recognition reaction. These differences will be used to develop a PCR-based test to identify common incompatibility groupings.
The secondary goal will be investigated using confocal microscopy to identify the nature and timing of the interaction between egg and sperm cells. The influence of hormone levels on this interaction will also be investigated. The corrective influence of mentor pollination and the application of significant quantities of 'compatible' protein to mask an incompatibility recognition event will also be studied.

Timetable

Year 1

- Make a series of self-pollinations and cross-pollinations to identify clones most suitable for subsequent study.
- Develop protocol for the extraction of proteins/glycoproteins from pollen kit.
- Separate proteins using Isoelectric focusing or 2-D electrophoresis.
- Search database for candidate homologues described in other sporophytic systems.
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Year 2

- Make a series of self and cross-pollinations to fully characterise self-incompatibility and cross-compatibility of selected clones.
- Develop protocol for isolation and purification of proteins from pollen kit.
- Design primers from candidate homologues identified in search.

- Amplify homologous sequences in cocoa by PCR.
- Characterise the DNA sequence of PCR amplification products.
- Start to develop confocal methodology for investigating sperm-egg interaction in compatible and incompatible crosses.

Year 3

- Complete the series of pollinations to characterise self-incompatibility and cross-compatibility of clones for subsequent study.
- Investigate possibility of protein electrophoresis as an assay for incompatibility grouping.
- Identify amino acid sequence of proteins isolated from pollen kit.
- Design primers to PCR amplify relevant sequences from cocoa DNA.
- Determine the DNA sequence of amplified DNA.
- Design primers to exploit sequence differences between clones for proteins responsible for incompatibility recognition reaction.
- Develop PCR test to exploit DNA sequence polymorphisms identified from candidate homology study.
- Continue confocal study of sperm-egg interaction.

Year 4

- Complete development of PCR test for incompatibility groupings from isolated (pollen kit) proteins.
- Apply the test or tests developed to distinguish between incompatibility groupings on a range of clones with fully characterised self-incompatibility and cross-compatibility relationships.
- Select the best test or tests for characterisation of germplasm collection.

Year 5

- Complete confocal study of egg-sperm interaction in response to hormonal treatments.
- Screen through cocoa plants in the quarantine collection using the selected test(s) to characterise their incompatibility grouping.
- Write thesis for submission for degree of Ph.D.

Costs

	year 1	year 2	year 3	year 4	year 5
Research Assistant[1]	£10,680	11,439	12,374	13,374	14,265
Supply of cocoa material[2]	£ 800	800	800	300	300
Consumables	£ 2,500	3,000	3300	3000	2500
Overheads	£ 4,272	4,576	4,950	5,350	5,706
	£ 18,252	19,815	21,424	22,024	22,771

[1]Based on point 6 of the RNA scale as at October 1998 with an annual increment and an estimated % annual increase. It is assumed that the BCCCA will meet any nationally agreed pay awards during the course of the project.

[2]Cocoa material will be supplied/grown for experimental purposes by the Reading Intermediate Quarantine Facility

Project proposed by Dr M.J.Wilkinson