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par

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Characterization of a resistance locus to coffee leaf rust (*Hemileia vastratrix*) in coffee trees: genomic organization, diversity and development of tools for functional gene validation

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ABREVIATIONS

BAC Bacterial Artificial Chromosome
6-BA: 6-benzylaminopurine
CaMV: cauliflower mosaic virus
CC Coiled-Coil
CGA candidate gene approach
CG candidate gene
CLR Coffee Leaf Rust
CP medium: callus proliferation medium
DNA Deoxyribonucleic acid
2,4-D: 2,4-dichlorophenoxyacetic acid
ECP medium: embryogenic callus production medium
ETI Effector-Triggered Immunity
ETS Effector-Triggered Susceptibility
GFP *Green Fluorescent Protein*
HR Hypersensitive Response
HPTII hygromycin phosphotransferase II
i.e. id est
Ka non-synonymous substitutions
Ks synonymous substitutions
LRR Leucine-Rich Repeats
LTR Long Terminal Repeat
M medium: maturation medium
MAMP Microbe-Associated Molecular Pattern
MAS market assisted selection
NBS Nucleotide-Binding site
NPTII Neomycin Phosphotransferase III
OD: optical density
PAMP Pathogen-Associated Molecular Pattern
PCR Polymerase Chain Reaction
PEMs: proembryogenic masses
PTI PAMP-Triggered Immunity
QTL Quantitative-Trait Locus
R: regeneration medium
RLK Receptor-Like Kinase
RLP Receptor-Like Protein
SD: standard deviation
SNP single nucleotide polymorphism
TAIR: the Arabidopsis information resources
TIR Toll and Interleukin-1 Receptor

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General Introduction

Agriculture is extremely vulnerable to climate change. Higher temperatures can reduce crop yield and quality while increasing weed and pest proliferation. Crop protection against pathogens that cause epidemic diseases is a major asset for global food security and sustainable crop production. Using resistant cultivars remains the most ecological, economic and environmentally friendly method to grow a crop with limited pesticide applications and low production costs.

The genetic management of diseases requires the characterization of plant-pathogen interaction and as well as the mechanisms of co-evolution. Much progress has been made in understanding the organization and evolution of resistance genes in annual plants or short-lived perennial crops. On the other hand, little is known about the evolutionary history of these genes in woody perennial plants.

Coffee is the most popular beverage worldwide and an economic mainstay for many countries and about 25 million people. Brazil is the most important coffee-producing country being responsible for about a third of all world coffee production (International Coffee Organization, www.ico.org).

Coffee leaf rust caused by *Hemileia vastatrix* is the main disease affecting coffee plants and leads to significant reduction in productivity. Conventional coffee breeding is a very long process. At least 25 years are necessary to restore the genetic background of the recipient cultivar and thereby ensuring good quality of the improved variety. The source of disease resistance for coffee breeding comes from wild diploid coffee species and from the Timor hybrid, a spontaneous interspecific hybrid between *C. arabica* and *C. canephora*. However, during the last few years, improved varieties derivated from Timor hybrid are gradually losing their resistance to coffee rust due to the emergence of new virulent races.

The exploitation of genetic diversity using genomic approaches can significantly contribute for crop breeding by facilitating the localization and the access to important agronomical traits in the gene pool of wild coffee species. The detailed characterization of the resistance genes to pests and diseases, as well as technologies allowing the precise manipulation and exploitation of resistance genes could be useful in elaborating strategies directed to the development of cultivars exhibiting durable resistance to rust.

The present study relates the characterization of a multigenic locus conferring resistance to coffee leaf rust resistance. It was conjointly financed by IRD, CIRAD and the Brazilian government through CAPES foundation from the Ministry of Education of Brazil.

CHAPTER I

LITERATURE REVIEW

PART I

1. Generalities about Coffee

1.1 Coffee consumption, cultivation and economical trade

Coffee is the most popular beverage. It's consumed either hot or cold by about one-third of the people in the world. Its popularity can be attributed to its stimulant effect, which is produced by caffeine, an alkaloid present in green coffee in amounts between 0.8 - 1.4% for the Arabica varieties and 1.7 - 4.0% for Robusta (www.ico.org).

According to International Coffee Organization (www.ico.org) about 1.4 billion cups of coffee is consumed every day worldwide. The biggest consumers of coffee are the Nordic countries (Table I. 1). The leader in consumption is Finland with 12 kg per capita in 2007.

Coffee is cultivated between the Tropics of Cancer and Capricorn forming a "bean belt" (Figure I.1). Growing regions typically offer moderate sunshine and rain, steady temperatures around 20 ºC (70 ºF), and rich, porous soil. The coffee beans are an economic mainstay for many countries and about 25 million people. It is the second natural commodities; having a monetary value surpassed only by oil.

The total production for crop year 2009/10 totaled 120.6 million bags (www.ico.org). Brazil is responsible for about a third of all coffee production (39.4 million bags of 60 kg), making it by far the most important coffee-producing country, followed by Vietnam, Indonesia, Colombia with 18, 10.6 and 9 million bags of 60 kg, respectively in the crop year 2009/10.

Table I. 1 Top ten countries consumption of coffee ordered by annual *per capita* in 2007

Rank	Country	Coffee Consumption (kg per capita per year)
1	Finland	12
2	Norway	9.9
3	Iceland	9.0 (2006)*
4	Denmark	8.7
5	Netherlands	8.4
6	Sweden	8.2
7	Switzerland	7.9
8	Belgium/Luxembourg	6.8
9	Aruba	6.8 (2006)*
10	Canada	6.5

*Where 2007 data is not available, 2006 data is provided. Available online at: http://www.ico.org/historical.asp.

Figure I. 1 Major countries producers of coffee. Top ten coffee producers are shown in yellow

1.2 Coffee taxonomy

Coffee belongs to Rubiaceae family, one of the five largest flowering plants families, with aproximatly 13000 species classified in 620 genera, more than 40 tribes, and three subfamilies (Goevarts et al. 2006). Rubiaceae occurs on all continents but most taxa are in tropical or subtropical areas (Goevarts et al., 2006). Most of them are woody trees and shrubs. Plants of economical importance in this family include: Quinine (*Cinchona*) which possess medicinal properties, *Ixora* and *Gardenia*) cultivated as ornamentals plants, madder (*Rubia* sp) used as dyes and *Coffea* by far the most agronomical important species of Rubiaceae family.

The Rubiaceae family is divided into three subfamilies: Cinchonoideae, Ixoroideae and Rubioideae (Bremer & Eriksson, 2009). Ixoroideae subfamily that comprises about one-fifth of all Rubiaceae genera has 15 recognized tribes (Bremer & Eriksson, 2009), among them the tribu Coffeeae DC which comprises 11 genera including the genus *Coffea* and *Psilanthus* (Davis et al., 2007). The position of genus *Coffea* in angiosperms phylogeny is shown in Figure I.2.

The coffee species share the typical coffee bean morphology, i.e. a groove on the flat side of the seed. The 'husk' or 'parchment' (horny/ crustaceous endocarp) of the pyrene also has a deep ventral groove, which follows the invagination of the outer layer of the seed (exotesta) (Davis et al., 2006) (Figure I.3). They have been classified in two genera, *Coffea* L. and *Psilanthus* Hook. f. which differ in their flower morphology (Leroy 1980; Bridson 1987; Davis et al., 2005). The flowers in *Coffea* genus have long style, medium corolla tube, exserted anthers while in *Psilanthus* genus the flowers have short style, long corolla tube and encased anthers (Figure I.4).

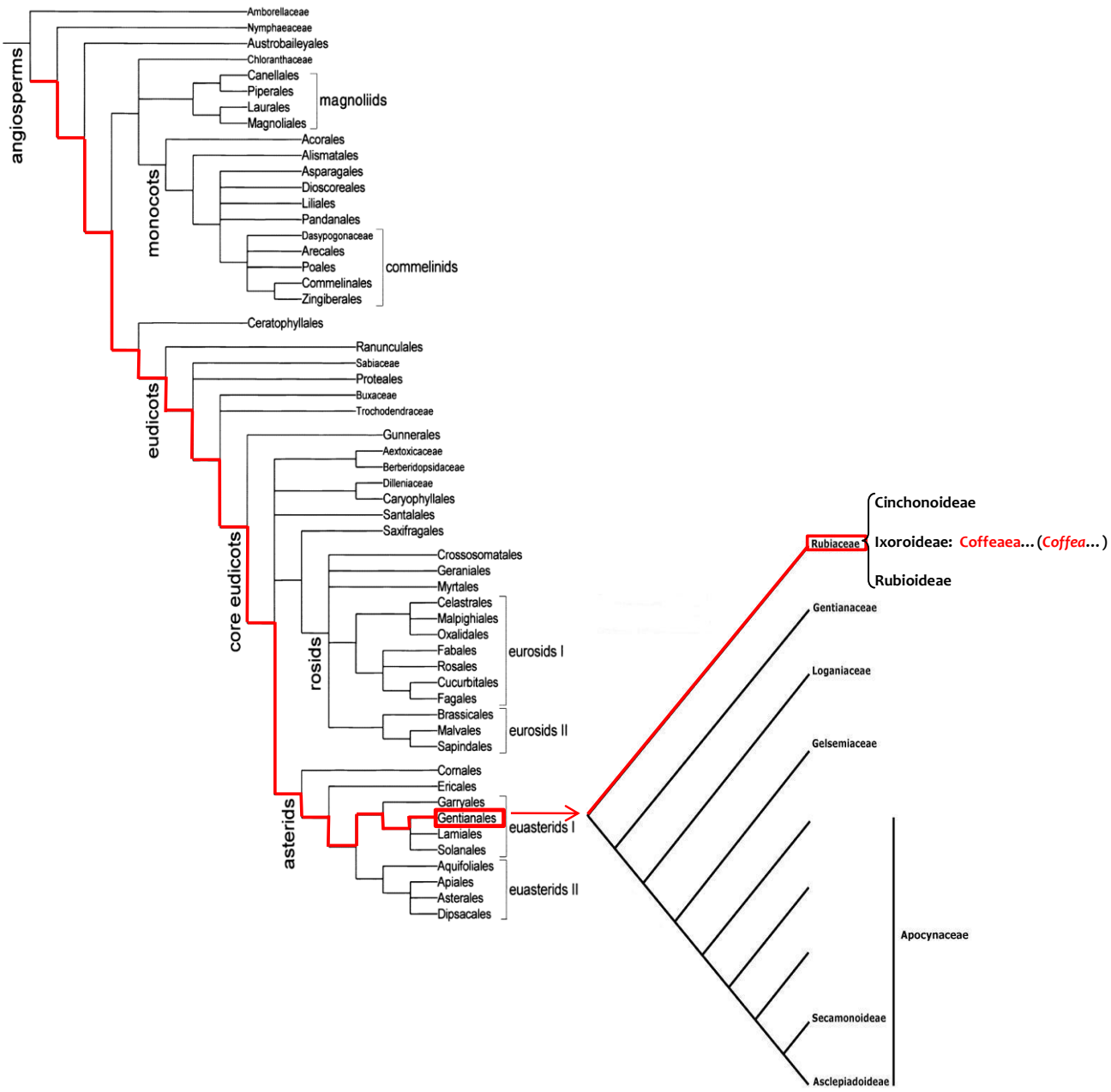

Figure I. 2 Position of genus *Coffea* on the angiosperms phylogeny. Modified from: (http://www.mobot.org/mobot/research/APweb)

Figure I. 3 Typical coffee bean morphology: A- Endocarp hard (horny/crustaceous), B- Seeds (and pyrenes) with a deep ventral groove.

Figure I. 4 Flower morphology in coffee genera. A. Coffea sessiflora in subgenus Coffea has flowers with long style, medium corolla tube, exserted anthers. B. Psilanthus mannii in subgenus Psilanthus has flowers with short style, long corolla tube, encased anthers.

The genus *Coffea* is classically divided in two subgenera: *Coffea* subgenus *Coffea* and *Coffea* subgenus *Baracoffea* (Bridson 2003; Davis 2003; Davis et al., 2005, 2006). However a sister relationship between subgenus *Coffea* and *Baracoffea* was demonstrated to be highly unlikely (Maurin et al. 2007). Nowadays the subgenus *Baracoffea* is referred as the 'baracoffea alliance' as a mean of recognition of nine *Coffea* species that are not yet clearly positioned on *Coffea* phylogeny but represent a strongly and morphological distinct lineage (Davis and Rakotonasolo, 2008).

The genus *Psilanthus* has also been divided in two subgenus: *Psilanthus* subgenus *Psilanthus* (two species) and *Psilanthus* subgenus *Afrocoffea* (Moens) (20 species) (Bridson 1988; Davis et al., 2005, 2006; Davis and Rakotonasolo 2008). Although coffee species has been described in two genera *Coffea* and ***Psilanthus*** the relationship between them is not completely understood yet. There are some evidences for accepting them as a single genus, such as morphological traits (Robbrecht and Puff, 1986; Davis et al., 2005), molecular characteristics (Davis et al., 2007; Maurin et al., 2007), low sequence diversity and indications of paraphyly (Lashermes et al., 1997; Cros et al., 1998). However, further molecular data are needed to fully resolve the relationship between *Coffea* and *Psilanthus* (Maurin et al., 2007).

The genus *Coffea* subgenus *Coffea* contains 95 species occurring throughout the natural range of the genus in tropical Africa, Madagascar, the Comoros and the Mascarenes (Reunion and Mauritius) and plus 9 species grouped in the "baracoffea alliance" that is suggested as a dry-adapted radiation of *Coffea* in Madagascar (Davis and Rakotonasolo, 2008). New taxa of *Coffee* are still being discovered (Stoffelen et al., 2009). A recent phylogeographic analysis of *Coffea* subgenus *Coffea* species using data on plastid sequences suggested that Lower Guinea could be the centre of origin of *Coffea* subgenus *Coffea* (Anthony et al., 2009).

Species included into the genera *Coffea* are self-incompatible with exception of the allotetraploid species *C. arabica* (Carvalho et al., 1991) and the diploid species *C. heterocalyx* Stoff (Coulibaly et al., 2002) and *C. anthonyi* (formerly *C.* sp 'Moloundou') found out in Cameroon and in Republic of Congo (Stoffelen et al., 2009), respectively. Only two species are of economical interest: *C. canephora* and *C. arabica*. The superior quality beverage is produced from *C. arabica* which is the most important traded species.

1.3 Characteristics of cultivated coffee species

The coffee plant is a woody shrub, and it grows in the wild as high as 12 meters, but cultivated trees are pruned to 2 meters. It has a main vertical trunk (orthotropic) and primary, secondary, and tertiary horizontal branches (plagiotropic). Small, white flowers give rise to a red or yellow fleshy fruit, the "coffee cherry" (Figure I.5) which contains a pair of beans (http://www.ico.org).

Figure I. 5 ***Coffea*** *arabica* tree with cherry beans. From: Leo Junior, Incaper ES

Coffea arabica

Coffea arabica is allotetraploid species. There are two distinct botanical varieties of arabica coffee: typica and bourbon. Historically, typica was cultivated in Latin America and Asia, whereas bourbon arrived in South America and, later, East Africa via the French colony of Bourbon (Reunion). Because *C. arabica* is mainly self-pollinating, these varieties tend to remain genetically stable. However, spontaneous mutants showing desirable characteristics have been cultivated in their own right, as well as being exploited for cross-breeding purposes. Some of these mutants and cultivars are described below: (summarized from Clifford and Willson, 1985).

Mutants: Caturra - a compact form of bourbon; Maragogipe - a mutant typica with large beans; San Ramon - a dwarf typica; and Purpurascens - purple leaved forms

Cultivars have been developed to give the maximum economic return under specific regional conditions such as climate, soil, methods of cultivation and the prevalence of pests and diseases. Some of the better known cultivars are: **Blue Mountain** - grown in Jamaica and Kenya; **Mundo Novo** - a cross between typica and bourbon, originally grown in Brazil; **Kent** - originally developed in India, showing some disease resistance; **Catuaí** - developed as a hybrid of Mundo Novo and Caturra, characterized by either yellow or red cherries: Catuai-amarelo and Catuai-vermelho respectively.

Arabica F1 hybrids

Arabica F1 hybrids deriving from crosses between wild Sudan-Ethiopian and American varieties and propagated in somatic embryo form have been created in Central America to increase the genetic diversity of coffee in the region (Bertrand et al., 2010). The yield of all hybrids is superior to the mean yield of American varieties and displayed better earliness. Also, the hybrids displayed better stability over environments than did the American varieties.

Coffea canephora

C. canephora is diploid and self-sterile, producing many different forms and varieties in the wild. The identification of cultivars is confused, but two main forms are recognized: **Robusta -** upright forms; **Nganda-** spreading forms (ICO). Two Canephora seed varieties have been selected for use as root-stock for Arabica with resistance to nematodes: Apoatã in Brazil and Nemaya in Central America. A brief comparison of botanical aspects between *C. arabica* and *C. canephora* is presented in Table I.2.

Table I. 2 Comparison of some botanical aspects between *C. arabica* and *C. canephora* species

Botanical aspect	*C. arabica*	*C. canephora*
Date species described	1753	1895
Chromosomes (2n)	44	22
Time from flower to ripe cherry	9 months	10-11 months
Flowering	after rain	after rain
Ripe cherries	fall	stay
Yield (kg beans/ha)	1500-3000	2300-4000
Root system	deep	shallow
Optimum temperature (yearly average)	15-24° C	24-30° C
Optimal rainfall	1500-2000 mm	2000-3000 mm
Optimum altitude	1000-2000 m	0-700 m
Hemileia vastatrix	susceptible	resistant
Koleroga	susceptible	tolerant
Nematodes	susceptible	resistant
Tracheomycosis	resistant	susceptible
Coffee berry disease	susceptible	resistant
Caffeine content of beans	0.8-1.4%	1.7-4.0%
Shape of bean	flat	oval
Typical brew characteristics	acidity	bitterness, full
Body	average 1.2%	average 2.0%

Available in: http://www.ico.org/botanical.asp accessed in 23 July 2010 00:06

1.4 Cytological aspects of *Coffea*

Cytological studies in coffee and other plants of Rubiaceae family made by Homeyer (1933 in Krug, 1934), demonstrated that *C. arabica* possess 2n=44 chromosomes while diploid species possess 2n=22 chromosomes. They also determined n=11 as a basic number of chromosomes for the genus *Coffea*. These results were confirmed later by Fagerlind (1937).

DNA content in plants and others eucariots are usually expressed in picograms (pg) or in Megabase pairs of nucleotides (Mb) where, 1 Mb = 10^6 nucleotide base pairs and 1 pg = 965Mb (Bennett and Leitch 1995).

The Chromosomal DNA 2C content (i.e a nucleus in G_1 phase of the cell cycle, with two copies of unreplicated genome) of *Coffea* species have been measured by flow cytometry (FCM) and slight variation has been reported by different authors depending on the methodology, standards, buffers or fluorochromes used. The mean of chromosomal DNA 2C content estimate for different cultivars of *C. arabica* ranged from 2.38 to 2.84 pg (Cros et al., 1995; Clarindo and Carvalho 2009) and from 1.32 to 1.76 pg for *C. canephora* cultivars (Cros et al., 1995; Noirot et al., 2003; Clarindo and Carvalho, 2009). The variations in DNA content among coffee species, besides of ploidy level (e.g. *C. arabica*), are probably due almost entirely to variation in the copy number of repeated DNA sequences. Differences may correspond to genomic evolution correlated with an ecological adaptation process (Lashermes and Anthony, 2005).

1.5 Speciation and genome features of C. arabica

The genome constitution and mode of speciation of *C. arabica* have been subjected to several investigations. Based on cytological observations and fertility of interspecific hybrids, *C. canephora* and *C. congensis* were suggested to have a common ancestor with *C. arabica* (Carvalho 1952; Cramer 1957). Furthermore, *C. congensis* can be considered as an ecotype of *C. canephora* in the light of the fertility of interspecific hybrids (Louarn, 1993) and the genetic diversity detected by molecular markers (Prakash et al., 2005). These phylogenetic assumptions were consolidated by a high homology found in the ITS2 region sequences of *C. arabica*, *C. canephora* and *C. congensis* (Lashermes et al., 1997). Regarding cpDNA that exhibited a maternal inheritance in coffee (Lashermes et al. 1996), *C. arabica* appeared to be similar to two species, *C. eugenioides* Moore and *Coffea* sp. "Moloundou" (Cros et al., 1998).

Restriction fragment length polymorphism (RFLP) markers in combination with genomic *in situ* hybridization (GISH) were used to investigate the origin of *C. arabica*. By comparing the RFLP patterns of potential diploid progenitor species with those of *C. arabica*, the source of the two sets of chromosomes, or genomes, combined in *C. arabica* was specified. The genome organization of *C. arabica* was confirmed by GISH using simultaneously labeled total genomic DNA from the two putative genome donor species as probes (Lashermes et al., 1999). These results clearly suggested that *C. arabica* is an amphidiploid (i.e. C^a E^a genomes) resulting from the hybridization between *C. eugenioides* (E genome) and *C. canephora* (C genome) or ecotypes related to those diploid species (Figure I.6) Results also indicated low divergence between the two constitutive genomes of *C. arabica* and those of its progenitor species, suggesting that the speciation of *C. arabica* took place very recently. Precise localization in Central Africa of the speciation process of *C. arabica* based on the present distribution of the coffee species appeared difficult since the constitution and extent of tropical forest varied considerably during the late quaternary period.

Furthermore, in spite of the close relationship among the two constitutive genomes, bivalents are observed in meiosis and *C. arabica* is considered to display a diploid-like meiotic behavior (Krug and Mendes 1940). Investigations suggest that homeologous chromosomes do not pair in *C. arabica*, not as a consequence of structural differentiation, but because of the functioning of pairing regulating factors (Lashermes et al. 2000a).

1.6 Coffee arabica genetic diversity and breeding

Genetic diversity in *C. arabica* appeared extremely reduced in comparison to the diversity observed in *C. canephora*. These results were demonstrated using different techniques to detected polymorphism in coffee species such as: cleaved amplified polymorphisms (Lashermes et al., 1996; Orozco-Castilho et al., 1996), RAPD (Orozco-Castilho et al., 1994; Lashermes et al., 1996; Anthony et al., 2001; Aga et al., 2003; Silvestrini et al., 2008), RFLP (Paillard et al., 1996; Lashermes et al., 1999; Dussert et al., 2003), AFLP (Lashermes et al., 2000a; Anthony et al., 2002), and SNPs or microsatellites (Mettulio et al., 1999; Combes et al., 2000; Anthony et al., 2002).

Comparing wild and cultivated Arabica coffee, genetic differentiation appeared demonstrated to be low between cultivated species (Typica and Bourbon groups) and to some extent high between wild and cultivated coffee (Lashermes and Anthony, 2005). This low genetic diversity has been attributed to the allotetraploid origin, reproductive biology, and evolution process of *C. arabica* (Lashermes et al., 2000). Hence wild coffee population could be used to enlarge the genetic base of cultivars (Lashermes and Anthony, 2005).

As a self-pollinating plant the most used method for Arabica is line selection in segregating natural or artificially created population, whereas Canephora breeding has mainly involved the selection of clones and of hybrids between clones (Eskes and Leroy, 2009).

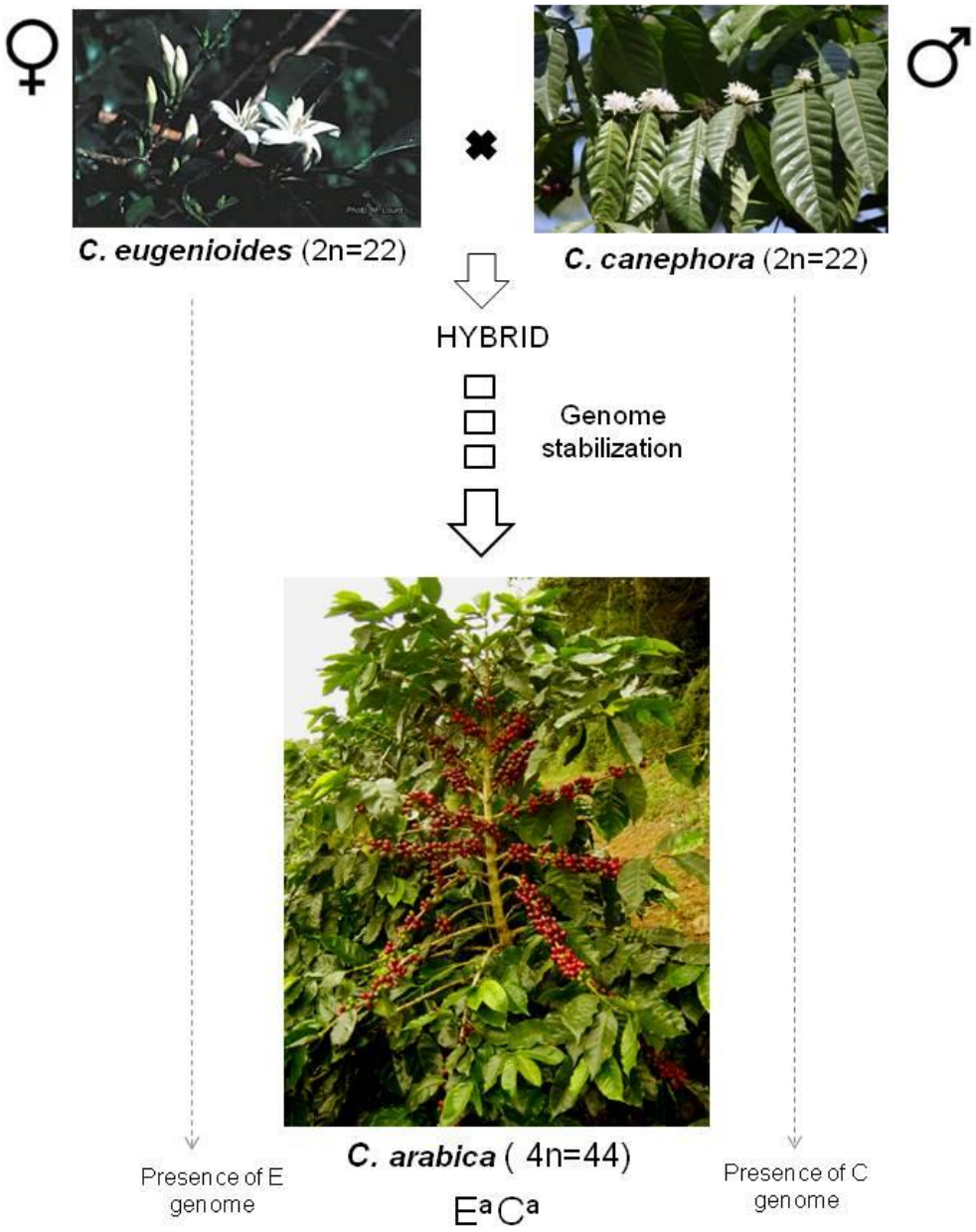

Figure I. 6 Genetic origin of allotetraploid *C. arabica*. Natural hybridization between *C. eugenioides* as female parent and *C. canephora* as male parent originated the *C. arabica* species (Lashermes et al., 1999).

The main strategy for Arabica breeding is to transfer genes desirable agronomical characters from diploid species into Arabica gene pool. Interspecific hybrids are produced by crossing and undesirable genes from the donor parent are gradually eliminated by selection. Many interesting agronomical traits have been identified in either spontaneous accession collected in the primary centre of diversity or diploid wild relative *Coffea* species (Table I.3).

Conventional coffee breeding face some difficulties such as: long generation time of coffee tree; high cost of field trial and lack of accuracy of current strategy. A minimum of 25 years after hybridization is required to restore the genetic background of the recipient cultivar and to ensure quality of improved variety (Lashermes et al., 2000).

Table I. 3 Examples of desirable agronomical characters identified in diploid species of coffee or in *C. arabica* wild type.

Desirable agronomical characters	Diversity source	Reference
Pest and disease resistance		
Perileucoptera coffeela	*C. racemosa*	Carvalho et al. 1988; Guerreiro et al.1999
Hemileia vastatrix	*C. canephora*	Berthaud & Lourd, 1982
	C. liberica, C. pseudozanguebarie	Kushalappa & Eskes, 1989
	C. liberica	Srinivasan and Narasimhaswamy, 1975
	C. eugenioides	Montagnon & Leroy, 1993
Colletotrichum kahawae	*C. canephora*	Rodrigues Jr. et al. 1975
Meloidogyne exigua	*C. eugenioides*, *C. racemosa*	Rodrigues Jr. et al. 1990
Meloidogyne paranaensis	*C. arabica* wild type, *C. canephora*	Van der Vossen, 1997
Meloidogyne incognita	*C. arabica* wild type, *C. canephora*	Bertrand et al. 1999; Anthony et al. 2002b
Meloidogyne arabicida	*C. arabica* wild type, *C. canephora*	Anthony et al. 2002b
Male sterility	*C. arabica* wild type	Dufour et al. 1997
Beans maturation	*C. congensis*, *C. canephora*	No reference
Biochemical seeds composition	*C. pseudozanguebarie*	Mazzafera & Carvalho, 1992
Adaptation to hydromorphic soils	*C. congensis*	Charrier & Vigreux, 1972
Drought tolerance	*C. racemosa*	Guerreiro-filho, 1972
Cold tolerance	*C. congensis*	Ahmad & Vishveshwara, 1980
Rusticity	*C. canephora*	Capot, 1977
Productivity	*C. canephora*, *C. arabica*, *C. liberica*	Capot, 1977; Carvalho et al. 1978
Beans mesh	*C. canephora*, *C. arabica*	Le Pierrès, 1995

1.6.1 Coffee breeding objectives

The mains objectives of coffee breeding are the creation and development of new varieties with the following characteristics (Eskes and Leroy, 2009):

- High-yielding varieties;
- Adaptation to different cultivations systems: dwarf coffee varieties and high density planting;
- Physiological disorders: drought resistance
- Increase resistance to pest and diseases such as: CLR (*Hemileia vastatrix*), CBD (*Colletotrichum kahawae*), *Fusarium, Cercospora coffeicola,* nematodes (*Meloidogyne, Pratylenchus* spp); insects: coffee leaf miner (*Leucoptera* spp), coffee berry borer (*Hypotenemus hampei*);
- Improve beverage quality: selection for low caffeine content, flavor features

1.7 Interspecific hybridization and coffee introgression

Introgressive hybridization was first defined as the infiltration of germplasm of one species into another through repeated backcrossing of hybrids to parental species (Anderson and Hubricht, 1938).

Occurrence of spontaneous hybrids between tetraploid arabica and other diploid species is common especially when these species are cultivated together (Prakash et al., 2002). Most of the disease resistance genes used in coffee breeding have been transferred into *C. arabica* genome via natural or artificial hybridization involving *C. canephora* or *C. liberica* (Berthaud 1978; Bettecourt and Rodrigues 1988; Dublin et al., 1991; Wrigley 1995).

The most important natural hybrid is the Timor Hybrid (HDT) discovered on the Timor Island (ancient Portuguese colony) (Bettencourt 1973). HDT is originated from a spontaneous cross between *C. arabica* and *C. canephora* (Bettencourt 1973; Gonçalves and Rodrigues 1976; Lashermes et al., 1993; Orozco-Castilho et al., 1994). Later, it was suggested that the *C. canephora* progenitor at the origin of the Timor Hybrid came from the Congo basin (Lashermes et al., 2002). Several coffee-producing countries such as: Brazil, Colombia and Kenya have used HDT in their Coffee breeding programs as the major font of resistance to pests and diseases.

The level of introgression from *C. canephora* into lines derived from the HDT was estimated to represent from 8 to 27% of the *C. canephora* genome. In addition, introgression appeared not restricted to chromosome recombination (Lashermes et al., 2000).

Other spontaneous hybrids between *C. arabica* and *C. canephora* have been identified in New Caledonia (HNC). Fourteen hybrids HNC have been recently investigated and some of them have exhibited a low number of introgressed markers reflecting a low proportion of *C. canephora* genome (i.e. 4%)(Mahé et al., 2007). Rust inoculation test demonstrated that all HNCs except one appeared resistant to the rust Race II. Individuals showing a low level of introgression could be directly used in breeding programmers' and significantly contribute to the development of improved Arabica cultivars exhibiting a valuable resistance to rust (Mahé et al., 2007).

Natural coffee hybrid discovered in India and named S.26 has been used in coffee breeding programs in India as main source for rust resistance (Vishveshwara, 1974a; Sreenivasan et al., 1994). These early Indian selections carry S_H3 resistance factor for coffee rust, only known to be present in *Coffea liberica* (Wagner & Bettencourt, 1965). So it was supposed that *C. liberica* was likely involved in the origin of this natural hybrid. To investigate this hypothesis AFLP markers were used to evaluate the introgression of *C. liberica* into S.288 population (derived from S.26) and 17 introgression lines derivate from a cross between S.288 x Kent variety (pure Arabica) (Prakash et al., 2002). The AFLP profiles were compared to 5 accessions of *C. liberica* and *C. arabica*. They found that introgression bands were associated with *C. liberica* var *liberica* (Guinean origin) and that introgression on *C. arabica* from *C. liberica* was restricted to few chromosomes segments. They assumed that S.26 might have originated either from a natural tetraploid F1 progenitor (union of unreduced gametes) or triploid F1 progenitor (union of reduced gametes) (Prakash et al., 2002).

The production of artificial interspecific triploid (2n=33) or tetraploid (2n=44) hybrids can be also a strategy to introduce genes from diploid coffee into *C. arabica* (Figure I.7). Triploid hybrids are derived from a direct cross between the diploid progenitor and *C. arabica* while tetraploid hybrids are obtained by crossing both species after chromosome duplication of the diploid coffee (Lashermes et al., 2000). A tetraploid hybrid *C. arabica* x *C. canephora* was created in Brazil in 1950. This hybrid was then back-crossed with Arabica cultivars resulting in the Icatu breeding population. Other hybrids called 'Arabusta' (*C. arabica* and *C. canephora*) or Congusta hybrids (*C. canephora* and *C. congensis*) were created in Ivory Coast, Cameroon and Ghana in order to develop varieties with better beverage quality of coffee and adapted to tropical lowlands (Eskes and Leroy, 2009).

In Colombia, triploid hybrids between *C. arabica* and *C. canephora* have also been created and used for further selection since the 1970s. Back-cross generations from hybrids between *C. arabica* and *C. racemosa* are being selected for resistance to coffee leaf miner in Brazil (Eskes and Leroy, 2009).

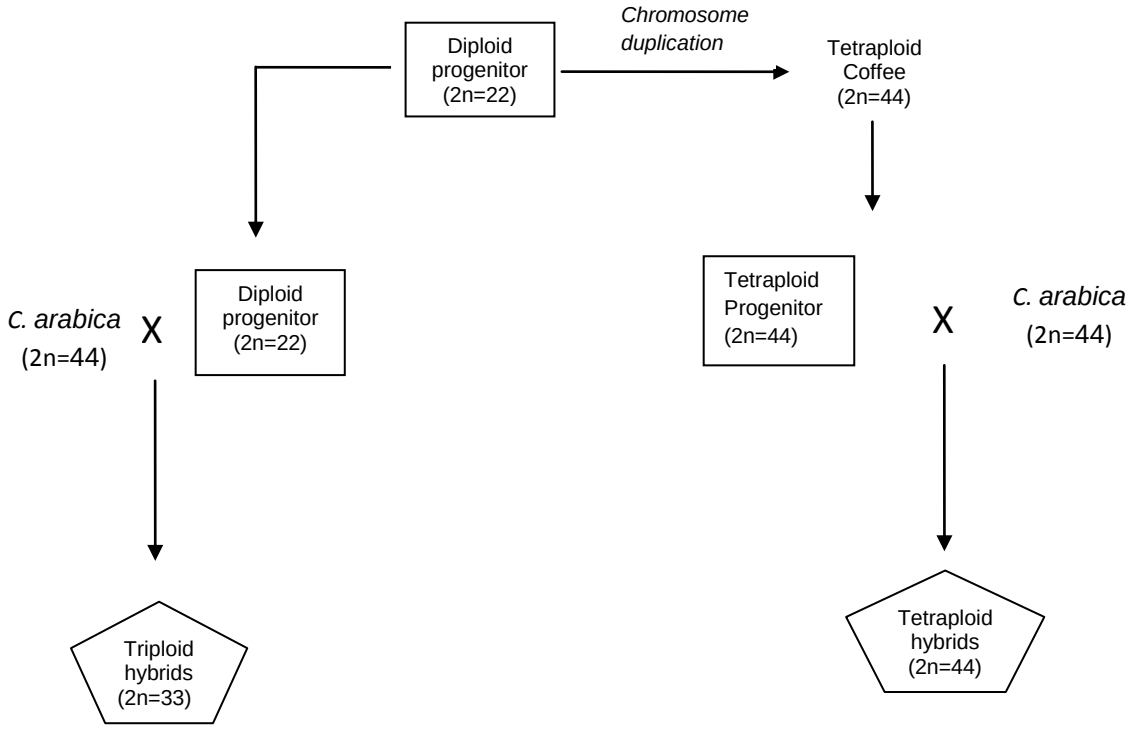

Figure I. 7. Triploid and tetraploid strategies of gene introgression from diploid coffee species into *C. arabica* genome. From: Lashermes et al., 2000.

2. Coffee leaf rust: the main coffee disease

2.1 The genus *Hemileia*

Coffee leaf rust is caused by a biotrophic fungus *Hemileia vastatrix* (Berk & Br.) that belongs to the class Basidiomycetes, the order Uredinales, and the family Pucciniaceae (Figure I.8). The coffee leaf rust is generally conceded to be endemic in the mountains of Ethiopia, the primary center of *C. arabica* diversity. Rust also occurred in wild coffee in the lowlands around the Lake Victoria, in Uganda and Kenya where it was first reported in 1861. The fungus was described associated with the disease on some dried coffee leaves by Berkeley in 1869 (Schieber and Zentmyer, 1984). The name *Hemileia* given to the genus refers to one half of the spores' cell wall which has a smooth aspect; the word *vastatrix* refers probably foreseeing its quick and through dissemination in the regions it would infest.

H. vastatrix is an obligatory parasite (i. e depends on live cell host for completion of their life cycle) specific of coffee plants (*Coffea* sp.) and some *Psilanthus*. Coffee leaf rust is one of the most

destructive diseases of crop *Coffea arabica*. The fungus is widely distributed in most part regions of the world where coffee is grown and can represent serious limitation to economically sustainable coffee production (Bettencourt and Rodrigues, 1988). The fungus has not been still detected in Hawaii and Australia (Van der Vossen et al., 2005).

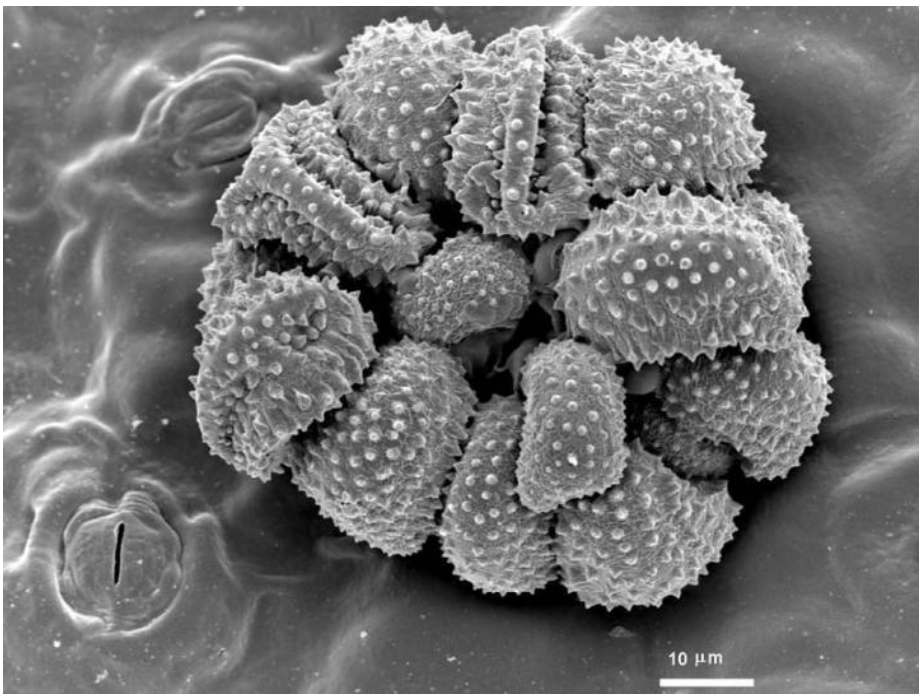

Figure I. 8 Urediniospores of *Hemileia vastatri*x. They are reniform-like, 26–40 × 18-28 µm, 1–2 µm thick, strongly warted on the convex side, smooth on the straight or concave side, warts frequently longer (3–7 µm) on spore edges. Photo from: Liberato JR & Silva MC (2006). Available online: http://www.padil.gov.au**.**

2.2 Coffee leaf rust (CLR) symptoms

H. vastatrix is macrocyclic rust with no alternate host so far identified. As other biotrophic fungus, the infection process of *H. vastatrix* on coffee leaves involves specific events including appressorium formation over stomata, penetration into the leaf and colonization of living host cells by intracellular specialized fungal structures (haustoria) that penetrate the plant cell wall and allow nutrient uptake. The life cycle of *H. vastatrix* is shown in Figure I.9.

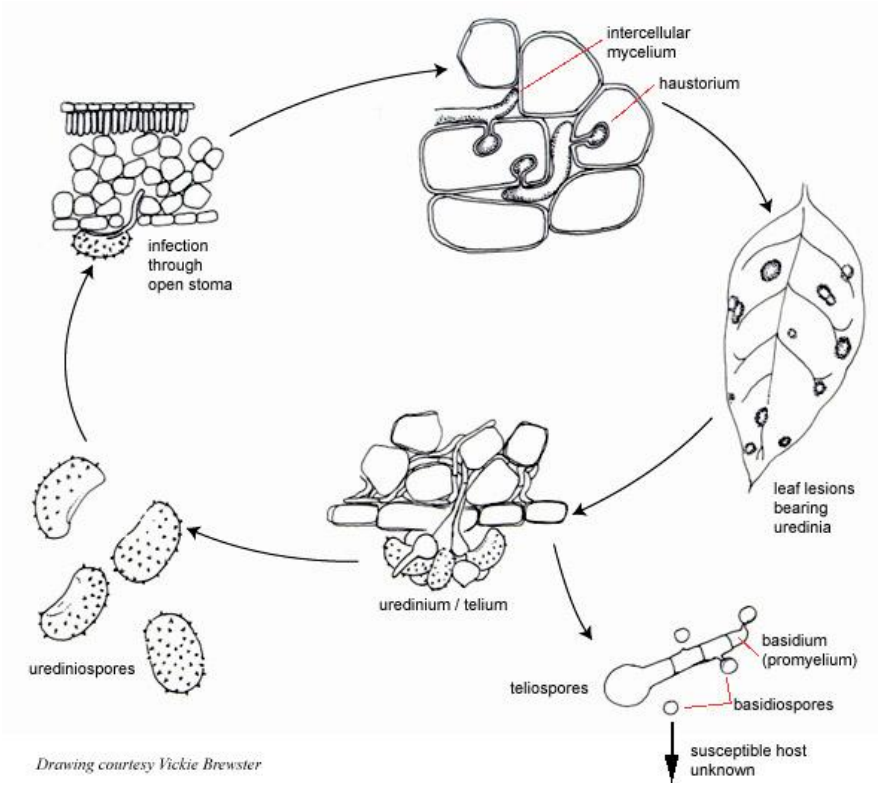

Figure I. 9 Life cycle of *Hemileia vastatrix*. Drawing from: (www.apsnet.org/online/feature/edcenter).

The first symptoms of the disease consist of the appearance of small light yellow spots of 1-3 mm diameter and with a slightly oily aspect on the leaf underside, when observed by transparence. These spots develop within a few days, become yellow orange and powdery and reach a diameter of 2-3 mm. When they coalesce, they can cover a great extent of the limb. The yellow-orange powder consists of the pathogen spores and is the only extreme sign of the disease. The attacked leaves generally drop prematurely, prejudicing the plant development and affecting the producing trees (D'Oliveira, 1954-1957; Chaves et al., 1970; Galli, 1970) (Figure I. 10). Little molecular information is available about *H. vastatrix*. Genetic diversity assessed by RAPD in isolates of *H. vastatrix* was considered moderately low and showed that rust isolates classified in the same physiological race by means of conventional methods (e.g., isolates from races XXIII and XXXVII) seemed to differ substantially at the molecular level. This variability could indicate the occurrence of genetic changes independent of pathogenicity (Gouveia et al., 2005).

A collaborative project involving INRA, IRD and the Genoscope to identify the virulence factors in Hemileia vastatrix are now in progress (http://www.genoscope.cns.fr/spip/Identification-of-virulence). The aim of this project is to use the 454-pyrosequencing technique to identify transcripts coding for rust virulence. Comparison of expressed sequence tags with genome sequences and catalogs of predicted genes available for plant and fungal species in international genomic databases

will allow deriving specific sequences belonging to the plant host or the pathogen. A particular attention will be given to transcripts encoding small secreted fungal proteins that likely contain putative effectors. This project is a very important step in improve knowledge about the mechanisms that control rust disease development in coffee plants.

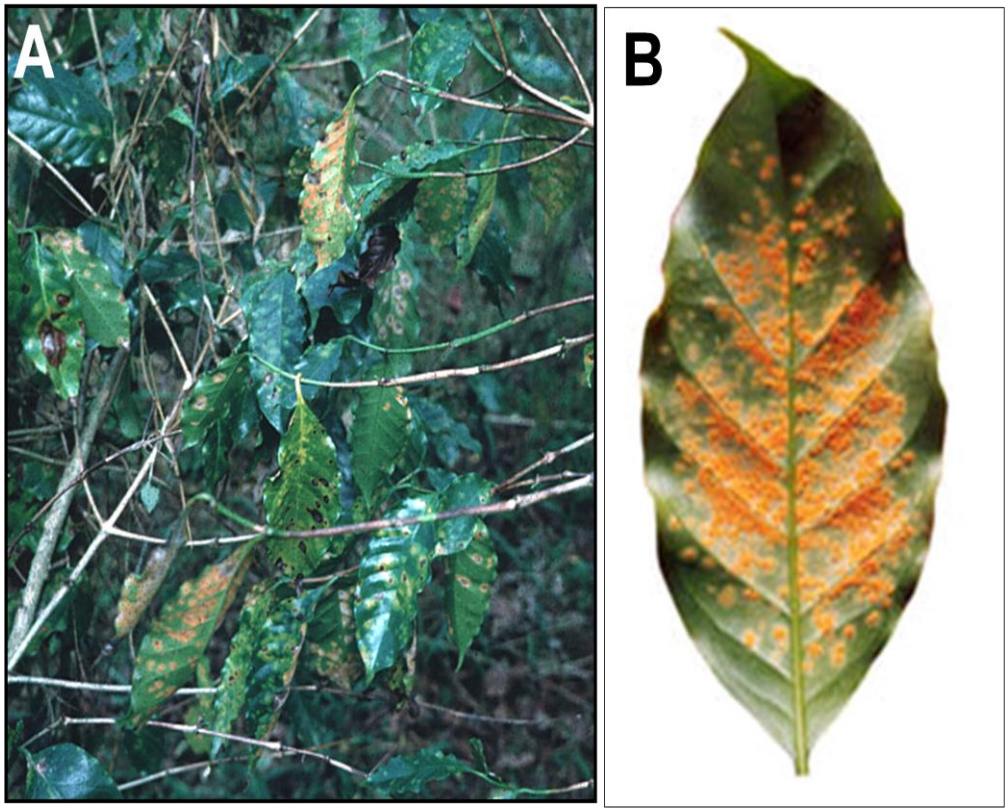

Figure I. 10 Symptoms of coffee leaf rust. A - coffee plants highly infected showing drop of leaves. B Detail of coffee leaf with yellow-orange powder urediniospores. Photo A from: (www.apsnet.org/online/feature/edcenter) and photo B (www2.iict.pt/index).

2.3 CLR epidemic

The CLR epidemic began in Ceylon (now known as Sri Lanka) in 1867 and quickly spread to other countries. Many factors led up to the epidemic, one of the most important was the destruction of native jungles in Ceylon and the establishment of an agricultural monoculture of coffee. The destruction of the agricultural base also led to economic problems and some affected countries switched to tea production as a source of income. Coffee rust was restricted to the Eastern Hemisphere

until 1970 when it first appeared in Brazil. Since that time it has spread to other coffee producing areas in South and Central America as well as Mexico (Schieber & Zentmyer, 1984).

When rust arrived in Brazil in the 1970, all cultivars in the Americas were susceptible. The cultivar Kent was the first useful resistant cultivar; it was developed on the Kent estate in Mysore, India. The Timor Hybrid, the diploid species *C. canephora* and *C. liberica* and the cv. Typica of Arabica were later incorporated in the breeding programs (Schieber & Zentmyer, 1984). (Figure I.11).

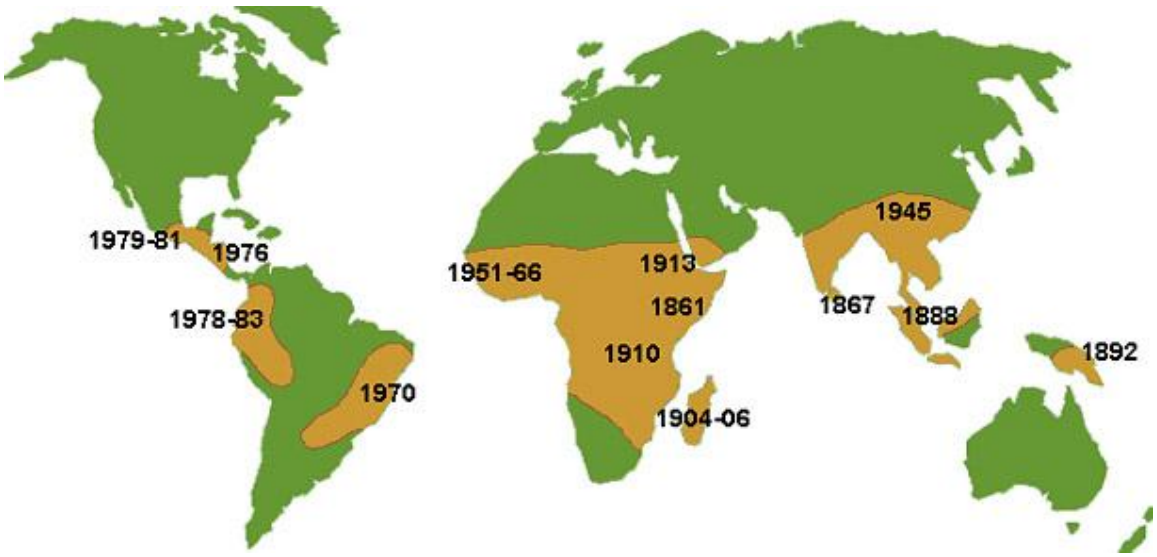

Figure I. 11 World distribution of coffee rust. http://www.apsnet.org/education/Lessonsplantpath/Coffeerust (Adapted from Schieber & Zentmyer, 1984)

2.4 Disease Management

Orange rust is the most harmful disease of the coffee-tree and it is responsible for heavy losses which correspond to millions of dollars for coffee culture in the world. The production losses due to rust are estimated at 30 - 70% in Brazil and India, respectively if no treatment is employed. Coffee rust causes premature defoliation, which reduces photosynthetic capacity and weakness the tree (Kushalappa and Eskes, 1989). Several factors may strongly influence coffee rust epidemics such as: shade status, coffee tree density, fertilization and pruning since they produce effects on microclimate and plant physiology which, in turn, influence the life cycle of the fungus. These factors should be considered in crop management depending if coffee is cultivated in intensive or extensive plantation (Avelino et al., 2004).

2.4.1 Chemical control

Copper-containing fungicides are very effective in controlling coffee rust. In *Coffea arabica*, the recommended number of fungicide applications in Brazil to control coffee leaf rust is four to five with copper fungicides or one to three applications of systemic fungicides from triazol group or triazo formulated with estrobilurins. In *C. canephora*, the number of foliar applications is usually half of number of applications done in *C. arabica* (Zambolin et al., 2005).

Some disadvantages in use of copper-containing fungicides include accumulation in the soil, particularly in the organic matter that can reach levels toxic to plants and to other organisms in the environment. Besides that, they must be present on the leaves before infection occurs. To minimize cost and to preserve environmental conditions chemical treatment should be applied only when the disease reach out a level of economic damage. For orange rust the application of fungicides is recommended when the disease incidence reach 5% (5% of infected leaves) (Almeida et al., 1986, Rivillas Osorio et al., 1999).

2.4.2 Biological control

Some researchers are focus on found out biologic agents to control coffee leaf rust, mainly for organic crop systems. A significant reduction in leaf rust severity by the presence of *Verticillium psalliotae*, especially when was applied 24 hours before the inoculation of *H. vastatrix*, demonstrated an evidence parasitism *in vivo* (Mahfud et al., 2006). It has been showed that the hyperparasitic fungus *Verticillium hemileia* is able to colonize the coffee rust lesions and reduces the viability of the urediniospores, but it has very little impact on overall rust development (Eskes et al., 1991).

Pseudomonas and mainly *Bacillus s*pp. were efficient in reducing the intensity of leaf rust under field conditions as effectively as copper hydroxide but many studies, including toxicological ones, are still required to prove the effectiveness and the safety to use these agents in biological control of CLR (Haddad et al., 2009).

2.5 Resistance to CLR

The use of resistant cultivars is the most efficient, economic and ecologically correct control for coffee leaf rust disease. Coffee-rust interactions can be expressed as immunity (total absence of symptoms) or susceptibility (formation of large pustules with abundant sporulation) (Bettencourt, 1981). The most common expression of resistance is the chlorotic dots or spots without spores. Chlorotic spots with little sporulation and small to medium-sized pustules correspond to intermediate expression of resistance may also occur (Varzea & Marques, 2005). For a number of coffee (*Coffea* spp.) genotypes, the resistance is post-haustorial (in that the fungus ceases its growth at different stages of the infection, but more frequently after the formation of the first haustorium) and is expressed by the rapid hypersensitive cell death (HR). Cell death began to be observed around 2 days post-inoculation, in the guard cells only, or in both the guard and subsidiary cells at the infection sites in which the fungus reached the stage of appressorium or penetration hypha (Silva et al., 2000, 2002).

Coffee-rust interations seem to follow the model proposed by Flor (1942, 1971) who was working with rust (*Melampsora lini*) of flax (*Linum usitatissimum*). He showed that the inheritance of both resistances in the host and parasite is controlled by pairs of matching genes. In this model, there is one gene in the plant called R gene and other in the parasite called *Avr* gene. Plants producing a specific R gene product are resistant towards a pathogen that produces the corresponding *Avr* gene product.

Recently, histological observations in compatible and incompatible coffee-rust interactions identified a biphasic haustorial differentiation of the *H. vastatrix* race II in both compatible and incompatible interactions (Ramiro et al., 2009). In the first phase, pioneer haustoria were formed in the subsidiary and adjacent cells of stomata from a first described infection structure named the pioneer hypha. In the second phase, secondary haustoria were produced in the mesophyll after differentiation of the anchor-shaped substomatal vesicle. In addition, specific resistance genes were expressed after differentiation of the *H. vastatrix* secondary haustoria, showing no evidence of specific recognition of coffee rust at the pioneer haustoria stage, suggesting that haustoria components are not recognized by, or not secreted into, the subsidiary and adjacent cells of the stomata (Ramiro et al., 2009). Brief, the authors also suggested that Avr proteins from *Hemileia vastatrix* race II are not specifically recognized by, or not secreted into, the epidermal cells of the stomatal region from pioneer haustoria.

2.5.1 CLR resistance genes

Resistance to coffee leaf rust in *C. arabica* appears so far conditioned primarily by 9 dominant major named S_H genes (identified so far) single or in association (S_H1-S_H9) while in *H. vastatrix* 9 genes for virulence named v1-to-v9 are in correspondence. The term S_H means "Susceptibility to Hemileia" in other word they are dominant genes that condition total susceptibility to compatible races and specific resistance to incompatible races (Rodrigues et al., 1975). In this way, for example coffee plants carrying the SH3 resistance factor are expected to manifest susceptibility when infected by races of *H. vastratrix* carrying the corresponding v3 virulence factor (Prakash et al., 2004).

Four dominant genes were characterized: S_H1, S_H2, S_H4 and S_H5 in *C. arabica*. The resistance factor SH3 was identified in the Indian selections derived from tetraploid coffee S.26 and S.31 (*C. arabica* x *C. liberica* (Noronha-Wagner & Bettencourt, 1967; Bettencourt & Noronha-Wagner, 1971) while the resistance factors (S_H5 – S_H9) were characterized in HDT derivatives and its supposed coming from *C. canephora* (Bettencourt, 1980; Bettencourt et al., 1992). Besides these SH genes identified at CIFC, it is likely that other major genes on HDT derivatives condition the *Coffea*-rust interactions (Bettencourt & Rodrigues, 1988, Eskes et al., 1990).

Screening for resistance and rust races has suggested that the factors S_H1, S_H2 S_H3 S_H4 and S_H6 appears to be dominant and monogenic (Rodiguez et al., 1975). The S_H4 gene may however be partially dominant or even quite recessive. No inheritance studies have been published yet on SH5. The inheritance of S_H7 to S_H9 appears quite complex when using races showing incomplete virulence, which results in intermediate resistance level in the progenies derived from crosses inoculated with such races (Eskes, 1989; Eskes et al.,1990).

Coffee genotypes may react as R (resistant), MR (moderately resistant), MS (moderately susceptible), or S (susceptible) to different rust races, which suggests that more than two levels of virulence may be present in relation to known resistance factors. The differential for SH3, for example, reacts like R, MS, or S to different races (Rodrigues et al., 1975).

Intermediate virulence has also been demonstrated in relation to two resistant genes from *C. canephora* and also in Icatu (hybrids between *C. arabica* x *C. canephora*) (Eskes et al., 1990). Intermediate virulence in dikariotic rust fungi might be explained by incomplete dominance of avirulence alleles, by multiplies alleles for virulence, or by interallelic interactions. The occurrence of intermediate virulence indicates that the level of residual resistance of major genes, after they have been matched by virulent races, may depend on the rust genotype. The presence of such incomplete virulence would suggest that incomplete monogenic resistance might not be durable, as the fungus may overcome the resistance factor by stepwise increases in virulence (Eskes, 2005).

In Brazil, isolates with some virulence to S_H3 have been obtained from field plants with S_H3, which were classified as completely avirulent to S_H3 at CIFC. Thus four levels of virulence may exist, a hypothesis that indicates that either the virulence to S_H3 is more complex than expected from a gene-for-gene relationship or that the S_H3 factor is more complex than imagined (Eskes, 2005).

2.5.2 Physiological races of *Hemileia vastatrix*

The characterization of physiological races of *H. vastatrix* in relation to resistance to leaf rust has been made by CIFC (Rodrigues Jr., 1975; Bettencourt & Rodrigues, 1988; Varzea et al., 2002). It is based on phenotypic expression of different interactions between differential coffee varieties and the physiological races of rust. These groups are distinguished from each other essentially by reactions of complete resistance and susceptibility to several races of the pathogen and are named arbitrary by letters and numbers (Bettencourt, 1981) (Table I.4).

Up to now 38 physiologic groups and 45 physiological races have been characterized (Varzea et al., 2002) (Table I.5). For example, the Group A is characterized by resistance to all the known races and has been found in diploid species such as: *C. canephora*, *C. liberica*, *C. dewevrei*, *C. eugenioides*, *C. congensis* and *C. zamguebarie* (d'Oliveira & Rodrigues, 1961; Bettencourt et al., 1965) and in Hybrids between *C. arabica* x *C. canephora* (HDT, Icatu). No pure Arabica (typical and Bourbon cultivars) is present in this group. In the other hand, Group F represents susceptibility to all the known rust races. This group includes most accessions of *C. racemosa*. Between these extremities of "A" and "F" groups, there are 38 groups, ranging from susceptibility to 43 races to only 3 races (group W) (Bettencourt, 1981; Bettencourt & Rodrigues, 1985). Works at CIFC has demonstrated that the population classified as group A is not uniform; more than 5 new physiological groups became susceptible to 5 different rust isolates from India (Varzea & Marques, 2005).

Table I. 4 Coffee differentials to coffee leaf rust, genotypes for rust resistance, designation and coffee physiological groups (Bettencourt & Rodriguez 1988; Bettencourt, 1992)

Genotype for resistance	Coffee selection or cultivar CICF n°	Coffee physiological group
S_H?	Matari (849/1)	β
S_H1	Dilla & Alghe (128/2)	α
S_H1,4	134/4 – S12 Kaffa	I
S_H1,5	87/1 - Geisha	C
S_H1,6	H 468/23	5
S_H1,2,5	1006/10-KP532(pI31)	L
S_H1,3,5	H 153/2	Z
S_H1,4,5	635/3- S 12 Kaffa	W
S_H1,4,6	H 539/8	8
S_H1,5,6	H 538/29	7
S_H 1,2,3,5	H 150/8	V
S_H1,2,4,5	HW 17/12	O
S_H1,3,4,5	H 148/5	U
S_H 1,2,3,4,5	HW 18/21	S
S_H 2,5	DK &/6	D
S_H 2,3,5	34/13-S 353 4/5	H
S_H 2,4,5	H 152/3	Y
S_H 2,5,6	H 537/18	6
S_H 2,3,4,5	H 147/1	T
S_H 2,3,5,6	H 535/10	9
S_H 3,5	S288-23	G
S_H 3,4,5	H 151/1	X
S_H 3,5,6	H 581/17	10
S_H 4	S 12 Kaffa	γ
S_H 4,5	110/5-S4 Agaro	J
S_H 4,5,6	H 583/5	11
S_H5	Bourbon (63/1)	E
S_H5,6	H 440/7	4
S_H5,7 or S_H5,7,9[a]	7960/15	Without designation
S_H5,6,9	H 419/20	3
S_H5,8	H 420/2	2
S_H5,6,7,9	H 420/10	1
S_H5,6,7,8,9,?	HDT 832/1	A
S_H ?	Hybrid Kawisari	M
No S_H	*C. racemosa*	F
S_H6,	Catimor	R

[a]This genotype needs confirmation

Table I. 5 Genotypes for virulence in races of *H. vastatrix* pathogenic to *C. arabica* and some tetraploid segregants of *C. arabica* x *Coffee* spp (Beleonnur selections, Icatu and HDT derivates) characterized by CIFC (Varzea et al., 2002).

Physiological races	Genotypes for virulence
I	v2, 5
II	v5
III	v1, 5
IV	v?*
VI	v?*
VII	v3,5
VIII	v2,3,5
X	v1,4,5
XI	v?*
XII	v1,2,3,5
XIII	v5 ?*
XIV	v2,3,4,5
XV	v4,5
XVI	v1,2,3,4,5
XVII	v1,2,5
XVIII	v?*
XIX	v1, 4, ?*
XX	v?*
XXI	v?*
XXII	v5, (6)
XXIII	V1, 2, 4, 5
XXIV	V2, 4, 5
XXV	V2, 5, (6),?
XXVI	V4, 5, (6)
XXVII	V1, 4, (6)
XXVIII	V2, 4, (5, 6)
XXIX	V5, (6, 7, 8, 9)
XXX	V5, (8)
XXXI	V2, 5, (6, 9)
XXXII	V(6), ?
XXXIII	V5, (7) or v5, (7, 9)
XXXIV	V2, 5, (7) or v2, 5, (7,9)
XXXV	V2, 4, 5, (7, 9)
XXXVI	V2, 4, 5, (8)
XXXVII	V2, 5, (6, 7, 9)
XXXVIII	V1, 2, 4, 5, (8)
XXXIX	V2, 4, 5, (6, 7, 8, 9)
XL	V1, 2, 5, (6)
XLI	V2, 5, (8)
XLII	V2, 5, (7, 8) or v2, 5, (7, 8, 9)

*Unknown genotype for virulence, ( ) genes of virulence to HDT derivatives

3. Identification of molecular markers associated to CLR

The identification of molecular markers linked to R gene can be used in molecular marker-assisted selection (MAS) to accelerate incorporation and accumulation of such genes by pyramiding them into elite cultivars. Besides that, they are useful information for map-based gene cloning strategy.

AFLP markers have been used to detected rust resistance gene introgressed into *C. arabica* from *C. liberica* (Prakash et al., 2004). A population of F2 plants derived from a cross between two *Coffea arabica* inbred lines: Matari (high susceptibility to rust), and the *C. liberica* introgressed line S.288 (carrying S_H3 and S_H5 gene) was evaluated for resistance against three different races of *H. vastatrix*. The results revealed that the progeny segregated for the *SH3* gene in a 3:1 ratio, as expected for a single dominant gene (Prakash et al., 2004) (Table I.6).

The polymorphism analysis in this population using 80 different primer combinations revealed that at least half of the total polymorphism observed is associated with introgression of *C. liberica* chromosome fragments. Three genetically independent *C. liberica*-alien fragments in the genome of the introgressed line S.288 were identified, corresponding to a total length of 52.8 cM. The introgressed were estimated to represent a half-chromosome equivalent of *C. liberica* distributed over at least 3 chromosomes in the S.288 line (Prakash et al., 2004). This work was the first step towards exploitation of MAS and map-based gene cloning strategies in coffee using S_H3 genes.

In a subsequent study the same population used above was essayed to develop a genetic linkage map and for conversion of the identified AFLP markers into sequence characterized amplified region (SCAR) markers suitable for MAS (Mahé et al., 2008).

Table I. 6 Resistance segregation analysis in response to inoculation of three different races of *Hemileia vastatrix* An F2 population of 101 individuals derived from a cross between the susceptible line Matari and the line S.288 carrying the S_H3 and S_H5 rust resistance factor were evaluated (Prakash et al., 2004).

Plant resistance pattern		Response to different races (Resistance vs Susceptibility)			Frequency of F_2 individuals
Group	Resistance Genes	II (v_5)	VIII ($v_{2,3,5}$)	XIX ($v_{1,4}$)	(%)
A	S_H3, S_H5 or S_H3	R	S	R	78
B	S_H5	S	S	R	19
C	-	S	S	S	3

A total of ten sequence-characterized genetic markers closely associated with the S_H3 leaf rust resistance gene were generated. From these, three markers were linked in repulsion (i.e. linked in coupling with the susceptible allele from Matari), with the S_H3 gene and seven markers were clustered in coupling around the S_H3 gene. Two markers appeared to co-segregate perfectly with the S_H3 gene in the two plant populations analyzed and should be particularly appropriate for coffee MAS (Mahé et al., 2008).

The SCAR markers that appeared closed to S_H3 gene were used to screen a *C. arabica* BAC library previously developed by Noir et al (2004). Marker resources from a genetic linkage map of *C. canephora* involving microsatellite (i.e. simple sequence repeats, SSR) markers (Lashermes et al. 2001) as well as new markers derived from the positive BACs, were used to develop a genetic mapping of the S_H3 locus (Figure I. 12).

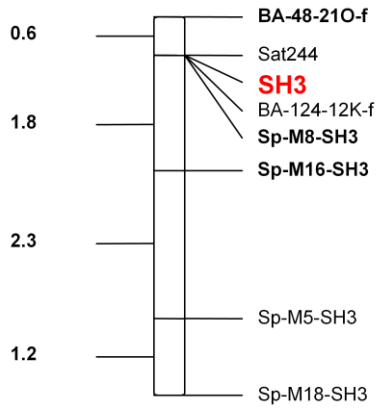

Figure I. 12 Linkage map based on analysis of the chromosome segments in coupling surrounding the S_H3 gene on the F2 population (Matari x S.288). Molecular marker names are on the right, while the estimated map distances in cM are shown on the left. SCAR markers showing codominant polymorphism are in bold (Mahé et al., 2008).

In situ hybridization techniques such as GISH and FISH have also been employed to detect the presence of alien fragments in the introgressed line S.288. Using total gDNA from *C. liberica* as probe in GISH, 8 hybridization signals corresponding to 4 introgressed fragments were detected in different chromosomes of S.288. Using AFLP only three introgressed fragments were detected (Prakash et al., 2004) while using GISH 4 introgressed fragments could be visualized. However, it is likely that AFLP marker screening failed to detect one relatively small introgressed fragment present in the S.288 genome (Herrera et al., 2007). GISH/BAC-FISH analysis was also used to confirm the presence of *C.*

liberica-introgression mediating disease resistance to leaf rust into distal position on chromosome 1 (Herrera et al., 2007).

In other study, the inheritance of coffee resistance genes to race II of *H. vastatrix* has also been analyzed by AFLP (Brito et al., 2010). The authors used a F_2 and BC (back crossing) population derived from a cross between the resistant genotype Timor Hibrid UFV 427-15 and the susceptible cultivar Catuai Amarelo UFV 2143-236. The segregation analysis showed that the resistance of this Timor Hibrid-derived genotype to race II of the *H. vastatrix* is conferred by a single dominant gene.

Three molecular markers linked to resistance gene were identified and the genetic mapping indicated that at least one molecular marker were linked to the resistance gene at 8.69 cM. The authors supposed that the identified resistance gene could be related to one of the genes S_H7, S_H8 or S_H9 or another unknown gene because the Timor Hybrid UFV-427-15 is also resistant to the race XXV which has, in addition to v2 and v5, the virulence allele v6.

The resistance inheritance to *H. vastatrix* races II and XXV in different accessions of Timor Hybrid has demonstrated to be governed by one to three genes depending on accession (Pereira, 1995). Those different patterns of inheritance are most likely reflecting the heterogeneity of the accessions derived from the original Timor Hybrid and distributed worldwide by CIFC in the 50's. In fact, the distribution was done by seed and genetic segregation obviously occurred (Bettencourt, 1973).

3.1 Partial resistance to CLR

To indentify polymorphic molecular markers associated with partial resistance to coffee leaf rust a segregating F2 population derived from a cross between the susceptible *Coffea arabica* cv. Caturra and a *C. canephora*-introgressed Arabica line (derived from HdT) exhibiting high partial resistance was analyzed (Herrera et al., 2009; 2010). Low level of alien introgression has been detected that could facilitate the rapid identification of putative resistant genes displayed by this genotype. Molecular analysis enabled identification of seven polymorphic markers (5 AFLP and 2 SSR) exhibiting significant association with partial resistance (Herrera et al., 2009). Segregation analysis in this population showed that although either two different markers confer its own rust resistance, added expression of resistance was also observed when resistance alleles co-existed at the two loci suggesting that these two loci could be involved in rust resistance and that their additive effect is essential for this resistance (Herrera et al., 2009).

PART II

1. Mechanisms of disease resistance in plants

Plants are naturally resistant to most pathogens because they have an innate ability to recognize potential invading pathogens and to activate successful defenses (Staskawicz, 2001). Plants differ from humans and animals because they do not have immune competent cells and circulation systems; plant cells protect themselves locally against infection (Kiraly et al., 2007).

Classically, the failure of most pathogenic microbes to infect plants is referred to as an ‘‘incompatible’’ interaction, and is based on two immune phenomena: nonhost and host-specific resistance (Hammond-kosack et al., 2003) (Figure I.13).

Non-host resistance is the first layer of plant innate immune system and is defined as strong and broad resistance to all members of a given pathogen species. In natural plant communities non-host resistance occurs when a plant is defeat by pathogens that have not co-evolved with the respective plant species (Schweizer, 2007). Non-host resistance is the most common and durable form of resistance and is known as basal resistance (Gimenez-Ibanez and Rathjen, 2010). On the other hand, host-specific resistance is the second layer of plant defense and dependent upon the presence of a particular pathogen race, a particular host plant cultivar, or both. The situation in which adapted pathogens can productively colonize certain host plants the (i.e. the ‘‘compatible’’interaction) is the exceptional case. During successful infections, the pathogen actively suppresses plant immunity (Gimenez-Ibanez and Rathjen, 2010).

1.1 Non host resistance

Non-host resistance against bacteria, fungi and oomycetes are classified into two types: type I and type II. The type I does not produce any visible symptoms (necrosis) and the type II is always associated with rapid localized necrosis (HR). During type I, the pathogen will not be able to get past the first or the second obstacle, and the multiplication and penetration into the plant cell will be completely arrested. The type II is the most commonly phenomenon of nonhost resistance is that produces a nonhost HR. This type of resistance is a more sophisticated plant defense mechanism than type I. Some pathogens can conquer early obstacles by producing detoxifying enzymes to overcome the toxic effect of preformed antimicrobial plant secondary metabolites (Mysore and Riu, 2004).

Interaction types	NON-HOST	HOST	
		INCOMPATIBLE	COMPATIBLE
Plant	Immune	Resistant	Susceptible
Pathogen	MAMPs detection - avirulence	Avr effector recognized-avirulent	Effectors/toxins interact with specific host target – virulent
Plant defense	Preformed structural or biochemical activation of innate immunity	Basal defense and R protein mediated activation of plant defense with cross-talk	Basal defenses only
Outcome	No disease-species incompatibility	No/highly reduced disease level Gene-for-gene mediated resistance	Disease – prolific pathogen replication and dissemination

Figure I. 13. Different types of plant-pathogen intecation (Hammond-Kosack & Kanyuka 2007).

Components of non-host resistance include: ***Pre-formed defense*** ex: plant actin microfilaments (cytoskeleton), secondary metabolites which can act as antimicrobial compounds such as: Saponins, steroid, or steroidal alkaloid molecules. ***Inducible plant defense mechanisms*** such as Phytoalexins that are synthesized in response to pathogens attack; ***Plant defense signaling*** like: hormone ethylene, salicylic acid, wound-induced protein kinase (WIPK) and salicylic acid-induced protein kinase (SIPK), Heat-skock proteins (Hsps), SGT1 (Skp1-cullin-F-box protein) ubiquitin ligase complex and ***broad-spectrum disease resistance genes:*** like *NHO1*, *EDS1*, *PEN1*, *PEN2*, *ROR2* (reviewed by Mysore and Riu, 2004).

Once the pathogens are able to overcome the pre-formed barriers, they also could be detected by the extracellular receptors of plant cell that are able to recognize some conserved structural components of potential pathogens (such as fungal chitin or bacterial flagellin) (Nurenberger et al. 2004). These conserved structural components are named pathogen/microbe–associated molecular patterns (PAMPs or MAMPs) and they are recognize through pattern recognition receptors (PRR) at the plant's cell surface leading to the induction of defense responses in plant (Jones and Dangl 2006;

Boller and He, 2009). The plant responses to PAMP/MAMPs have been called PAMP/MAMP-triggered immunity (MTI or PTI) (Jones and Dangl 2006; Boller and He, 2009).

1.1.1 PAMP/MAMP-triggered immunity (PTI/MTI)

MAMPs are elicitors with highly conserved molecular structures among whole classes of pathogens. They are indispensable for the microbial lifestyle and cannot be easily modified to evade recognition (Gimenez-Ibanez and Rathjen, 2010). Plants can recognize a wide number of bacterial P/MAMPs including flagellin, elongation factor Tu (EF-Tu), cold shock protein, peptidoglycan (PGN), lipopolysaccharides (LPS) and superoxide dismutase (Boler and Felix, 2009). The plant receptors that recognize P/MAMP are called Pattern Recognition Receptors (PRR) and are typically localized in the plasma membrane (Zipfel & Felix, 2005). The plant PRR so far known are: receptor kinases (RKs), receptor likeproteins (RLPs) (Shiu and Bleecker, 2001; Fritz-Laylin, et al., 2005) and carbohydrates (Zipfel, 2009).

Plant and animal cells can also recognize molecules from damaged host cells that are normally not available for recognition. These damage-associated molecular patterns (DAMPs) are released and recognized upon microbial attack (Lotze et al., 2007). Polysaccharides released from the plant cell wall (e.g. oligogalacturonides, or OGs), and some endogenous peptides are examples of DAMPs (Huckelhoven, 2007). The DAMPs seams to active the MAPK cascades similarly to the PTI ones, and they are also recognized by PRR, suggesting that MAMP and DAMPs have the same recognition pattern (Boller & Felix, 2009).

Pathogens that have the ability to cause infection on particular host plants have evolved means of suppressing of recognition of MAMP elicitors, through the use of effectors proteins that are delivered into host cells to manipulate these signaling processes. These effectors proteins are able of interfering with the host defense system function resulting in breakdown of host basal resistance establishing a basic compatibility between the pathogen and host (Dodds and Thrall, 2009). On the other hand, the selection pressure imposed by the successful pathogen lead the host plants to evolve novel defense mechanism to survive. This second mechanism of defense is called as host resistance or effector-triggered immunity (ETI), more commonly known as resistance (R) proteins that specifically recognize pathogen effectors and then trigger strong host defenses (Chisholm et al. 2006; Ellis et al. 2007; Bollar and He, 2009).

1.2 Host resistance (Effector-Triggered Immunity (ETI))

Effector-triggered immunity (ETI) also known as gene-by-gene theory was first proposed by Flor in 1947 when he studied the interaction between rust (*Melampsora lini*) of flax (*Linum usitatissimum*). He suggested that obligate parasites, such as the rust fungi, co-evolved with their hosts developing complementary genic systems. In this way, plants contain single dominant resistance (R) genes specifically recognize pathogens containing complementary avirulence genes. Specific recognition results in the induction of defense gene expression and the inhibition of pathogen growth. Nevertheless, if either R genes are absent in the plant host or R genes failed in recognize Avr genes, the pathogen is able to colonize it and cause disease on that plant. A diagram illustrating the gene-for-gene interaction using coffee-rust interaction as model is showed in Figure I.14.

Most R proteins control only pathogen races, which express the corresponding effector protein(s) and are therefore called race-specific R proteins. Occasionally, effective resistance is conferred against multiple races and even different pathogen species. These R proteins are called race-non-specific (Hammond-Kosak and Kanuya, 2007).

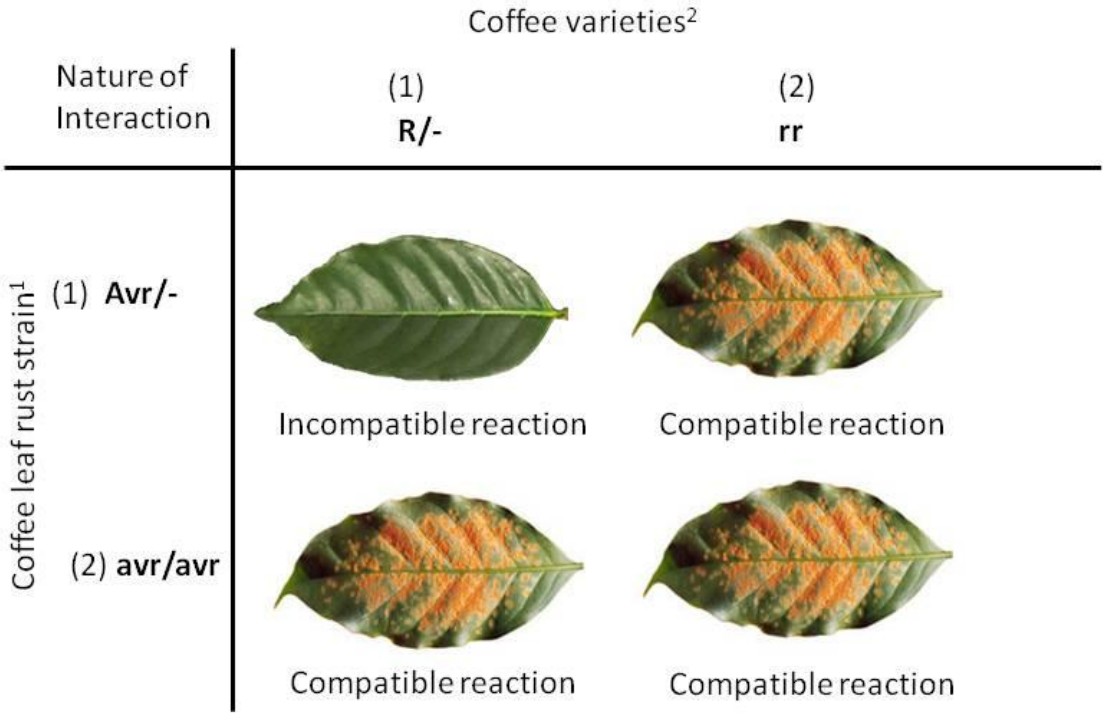

Figure I. 14 Quadratic diagram illustrating the gene-for-gene interaction between host resistance (R) genes and rust avirulence (Avr) genes using coffee-rust as model.

When there is an interaction between R gene and its corresponding Avr gene a type of programmed cell death termed the hypersensitive response (HR) occurs in the infected cells and immediate surrounding areas (Hammond- Kosack and Jones, 1996; Heath et al. 2000; Shirasu and Schulze-Lefert, 2000). R gene-mediated recognition of pathogen effectors activates a series of defense signaling cascades that involves protein phosphorylation, ion fluxes, generation of reactive oxygen species (ROS) and other signals (Hammond-Kosack and Jones, 1996; Yang et al., 1997; Somssich and Hahlbrock, 1998; Durrant and Dong, 2004). These signals subsequently trigger transcription of plant defense genes encoding proteins such as glutathione S-transferases, peroxidases, cell wall proteins, proteinase inhibitors, hydrolytic enzymes (e.g. chitinases and 1,3 glucanases), pathogenesis-related (PR) proteins and enzymes involved in secondary metabolism (Zhu et al., 1996). Although each branch of the plant immune system is controlled by distinct receptor classes, most of the downstream defense events associated with PTI and ETI are shared and it typical immune events activated upon microbial perception (Table I.7).

Table I. 7 Summary of the different forms and mechanisms of plant disease resistance (Kiraly et al., 2007)

Resistance phenomenon	Mechanism
Innate resistance	
Non-specific, gerenal resistence	
Non-host resitance	HR, ROS, BAX inhibitor-1, PEN genes
Basal resistance against bacteria	Flagellin/FLS2 interaction, ROS, antimicrobial compounds
Race non-specific mlo resistance and quantitative resistance to fungi	Cell wall thickening Antimicrobial compounds ROS
Resistance to necrosis-inducing stresses	High antioxidant capacity
Specific resistance (cultivar/pathogenic race specificity)	
Extreme resistance – symptomless gene-for-gene resistance Rx-resistance against viruses without HR Symptomless reaction to rust pathogens, no visible HR	Unknown
Gene-for-gene resistance R-gene « Avr-gene interaction associated with the hypersensitive response (HR)	ROS Phytoalexins Phenol oxidation Stress proteins
Resistance to pathogen toxins	Enzymatic detoxification Lack of toxin receptors
Gene silencing	Recognition and decomposition of foreign RNAs with ribonucleases
Acquired resistance	
After a primary infection an acquired resistance develops against a second infection 'Stress memory'	Accumulation of SA Stimulated antioxidants Gene silencing Rhizobacterial induction

Both PTI and ETI contribute to plant immunity and largely support nonhost and host-specific resistance, respectively, although the relationship is not exclusive. ETI is generally considered as an accelerated and amplified PTI response, resulting in disease resistance and, often, a hypersensitive cell death response (HR) at the infection site (Gimenez-Ibanez and Rathjen, 2010). The'zigzag' model was proposed by Jones and Dangl (2006) to represent four phases of the plant immune system (Figure I.15). In phase 1, PAMPs (or MAMPs) are recognized by PRRs, resulting in PAMP-triggered immunity (PTI) that can halt further colonization. In phase 2, successful pathogens deploy effectors that contribute to pathogen virulence. Effectors can interfere with PTI. This results in effector-triggered susceptibility (ETS). In phase 3, a given effector is 'specifically recognized' by one of the R proteins, resulting in effector-triggered immunity (ETI). Recognition is either indirect, or through direct R recognition of an effector. In phase 4, natural selection drives pathogens to avoid ETI either by shedding or diversifying the recognized effector gene, or by acquiring additional effectors that suppress ETI. Natural selection results in new R specificities so that ETI can be triggered again.

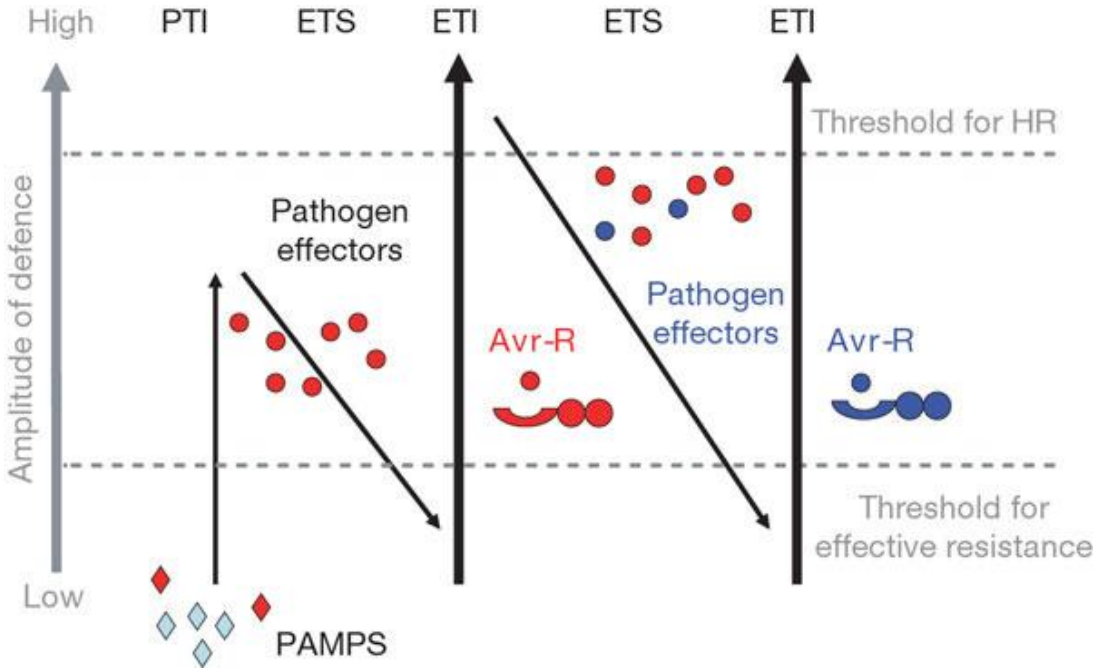

Figure I. 15 The'zigzag' model proposed to represent four phases of the plant immune system (Jones and Dangl 2006).

Brief, a simplified overview of the plant immune system for the post-invasive line of defense can be summarized as follow: First, the primary immune response recognizes common features of microbial pathogens, such as flagellin, chitin, glycoproteins and lipopolysaccharides. These microbial determinants are referred to as pathogen-associated molecular patterns (PAMPs). PAMPs activate pattern-recognition receptors (PRRs), which in turn initiate diverse downstream signaling events that ultimately result in the activation of a basal resistance that is called PAMP-triggered immunity (PTI; Figure I.16a). During the co-evolutionary arms race between pathogens and their host plants, pathogens acquired effector molecules that are transported into the host cell to suppress PTI and promote virulence of the pathogen, resulting in effector-triggered susceptibility (ETS; Figure I.16 b). In turn, plants acquired resistance (R) proteins that recognize these attacker-specific effectors, resulting in a secondary immune response called effector-triggered immunity (ETI; Figure I.16 c). Ultimately, the final outcome of the battle depends on the balance between the ability of the pathogen to suppress the plant's immune system and the capacity of the plant to recognize the pathogen and to activate effective defenses (reviewed by Pieterse et al., 2009).

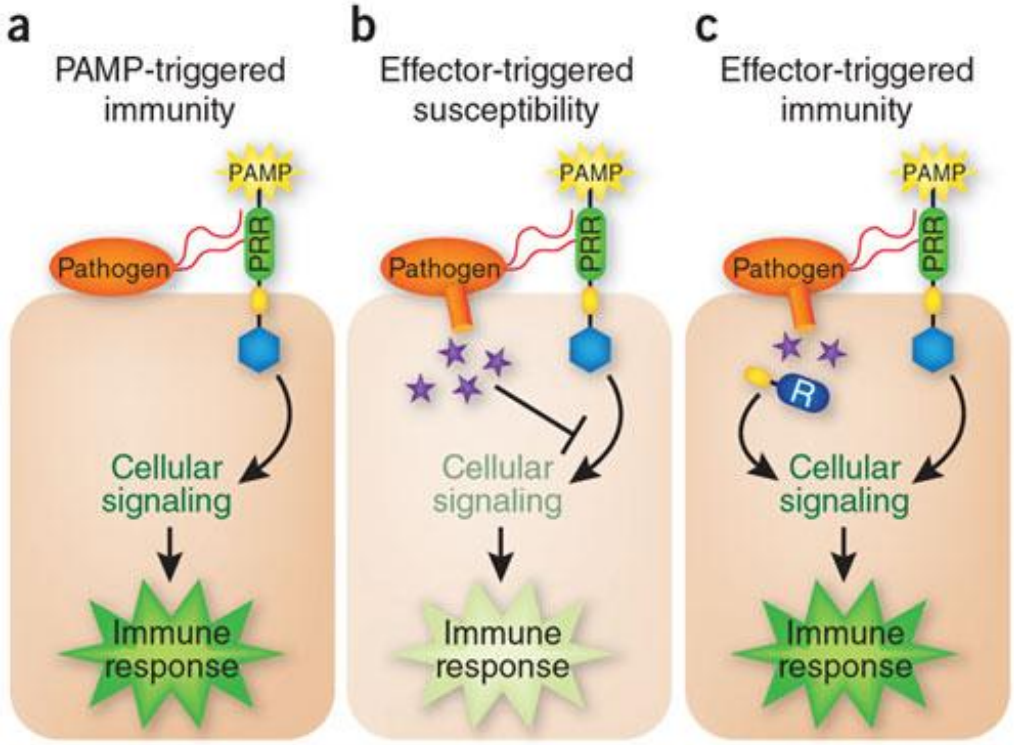

Figure I. 16 Simplified schematic representation of the plant immune system (Pieterse et al., 2009).

2. Modes of plant-pathogen interaction

2.1 Direct Interaction

The pathogen invading the plant cell can be recognized by R-Avr interactions that can occur direct or indirectly (Figure I.17). Direct interaction is the simplest form of recognition and occurs via direct physical association of the pathogen effector with the R immune receptor, similar to a ligand binding to its receptor (Caplan et al., 2008). In direct interaction an R protein detects an Avr protein and triggers host resistance, in this case selection pressure should be imposed on the pathogen carrying Avr gene to evade recognition that could occur by mutations in the Avr protein (Xiao et al., 2008).

Direct interaction has been demonstrated in the tomato Pto protein kinase, which is tied to the plasma membrane and interacts directly with its cognate bacterial effector AvrPto within the serine/threonine kinase activation domain at residue threonine 204. Tomato Pto also interacts directly with a second bacterial effector AvrPtoB, which has intrinsic E3 ubiquitin ligase activity. For three R proteins direct interaction with effectors has also been demonstrated. The Arabidopsis RRS1-R protein interacts with bacterial type III effector Pop2, rice Pi-ta interacts with AVR-Pita (a predicted secreted metalloprotease) from the Ascomycete rice blast fungus *Magnaporthe grisea* (reviewed by Hammond-Kosak and Kanuya, 2007).

The interaction between L locus from flax and cognate AvrL567 in *Melaspsora lini* was studied by Ellis et al. (1999). The *L* locus encodes at least 11 *R* alleles (including *L5*, *L6*, *L7*) capable of recognizing distinct *Avr* genes belonging to different loci, including *AvrL567* in the pathogen. Using the yeast two-hybrid system it was demonstrated that the R proteins L5, L6 and L7 physically direct interact with the corresponding Avr proteins. However, failure to detect direct R-Avr interactions in plants or in *vitro* in many R-Avr interactions prompted the researches to suggest existence of alternative recognition targets and even multiprotein recognition complexes such as Guard or Decoy model.

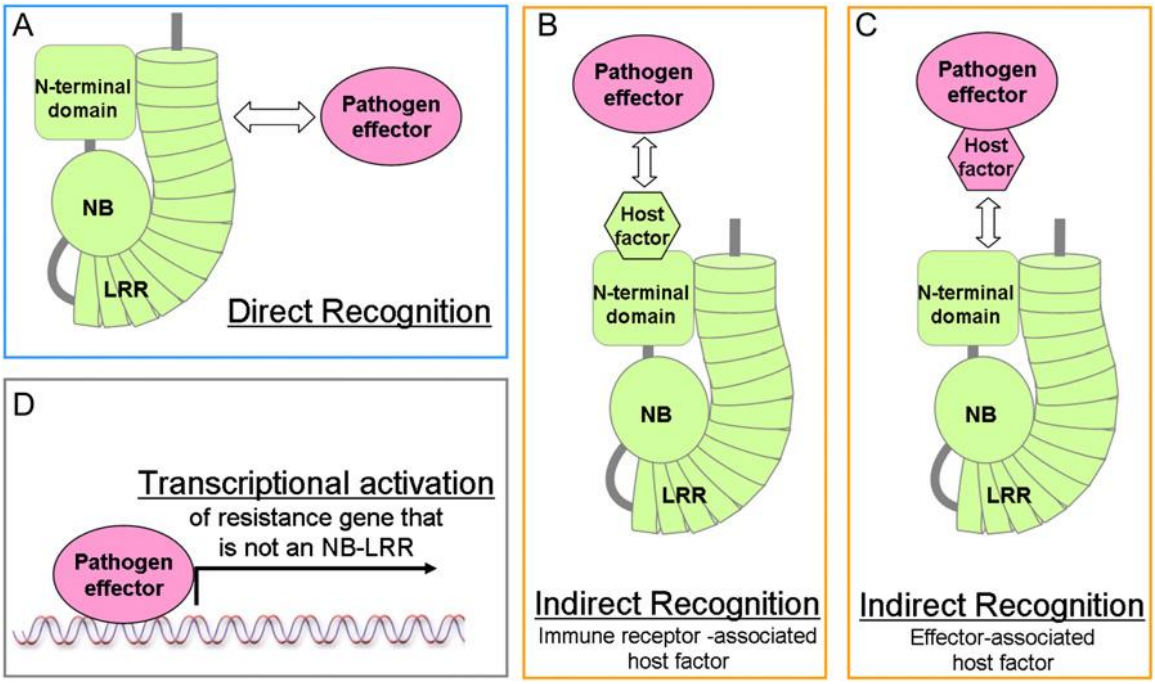

Figure I. 17 The three Modes of Pathogen Recognition. Direct interaction (A), Indirect interaction associated with the immune receptor (B) or with the pathogen effector (C) and then is subsequently recognized by the immune receptor. The third type of recognition occurs when a pathogen effector mimics a transcription factor and directly induces the expression of a non-NB-LRR resistance protein (D) (Caplan et al., 2008).

2.2 The Guard Model (indirect R-Avr) interactions

The guard model or 'guard hypothesis' suggests that host proteins targeted during pathogenesis are monitored, or guarded, by R proteins become cofactors in recognition (Van der Biezen, and Jones, 1998; Dangl and Jones, 2001). The Guard Model was originally proposed to explain the interaction between *Pseudomonas syringae* and AvrPto perception by the tomato proteins Pto and Prf (Van der Biezen and Jones, 1998). An example of guard model was characterized in Arabidopsis RIN4 protein where a host target for type III bacterial effectors is recognized by at least two CNL R proteins (RPM1 and RPS2). CNL protein RPM1 detects the phosphorylation of RPM1-Interacting Protein 4 (RIN4) by the pathogen effectors AvrB and AvrRpm1 from *Pseudomonas syringae* pv. *glycinea* and pv. *maculicola*, respectively, and elicits the resistance response (Mackey et al., 2002). In these instances RIN4 is a 211-amino acid acetylated protein, which is plasma membrane associated. AvrRpt2 is a cysteine protease and is a third effector that cleaves RIN4 at two sites recognized inside the plant cell (Axtell et al., 2003; Kim et al., 2005). Cleavage of RIN4 is detected,

however, by a second CNL the NB-LRR R protein, RPS2 that in turn elicits the defense response (Axtell & Staskawicz 2003; Mackey et al., 2003). Such indirect detection of pathogens allows a limited number of NBS-LRR R proteins to detect the activity of multiple pathogen effectors that target points of vulnerability in the plant (McHale et al., 2006). For example, tomato Mi-1, a CNL R protein, confers resistance to three species of root feeding nematodes as well as an aphid and a whitefly pest. Possibly, recognition of a common signal type or the guarding of a common host susceptibility target is involved in defense activation. Other *R* loci show dual specificity because of tightly linked genes with nearly identical sequences. An example is the neighboring genes in the potato genome, *Gpa2* and *Rx1* both of which encode CNL proteins and confer resistance to a nematode and a virus, respectively. *Gpa2*/*Rx1* protein domain swapping experiments have recently demonstrated that the recognition specificity is conferred by the C-terminal half of the LRR domain (reviewed by Hammond-Kosak and Kanuya, 2007). However, recent data on indirectly recognized effectors have emerged that are inconsistent with the original description of the Guard Model. Many pathogen effectors have multiple targets in the host and that classical guarded proteins are often dispensable for the virulence activities of effectors in plants lacking the R protein. New data on additional targets of AvrPto and AvrBs3 prompted proposals of the concept that some host targets of effectors act as decoys to detect pathogen effectors via R proteins (Zhou and Chai, 2008; Zipfel and Rathjen, 2008).

2.3 Decoy model

The decoy model is based on the supposition that selection pressures have favored recognition cofactors that continue to interact with Avr proteins but are not under pressure to avoid targeting by pathogen-derived proteins (Collier and Moffett 2009). The decoy mimics effector targets to trap the pathogen into a recognition event. Decoys might evolve from effectors targets by gene duplication followed by subsequent evolution or evolve independently by mimicking effectors targets (target mimicry). In any case, the Decoy Model implies that the effectors target monitored by the R protein is a decoy that mimics the operative effectors target but only functions in perception of pathogen effectors without contributing pathogen fitness in the absence of its cognate R protein (van der Hoorn and Kamoun 2008).

From an evolutionary point of view, the guarded effector target is in an unstable situation since it is subjected to two opposing natural selection forces in plant populations where *R* genes are polymorphic. In this case, *R* gene polymorphism means the presence/absence of functional *R* genes in different individuals in a plant population (Figure I.18).

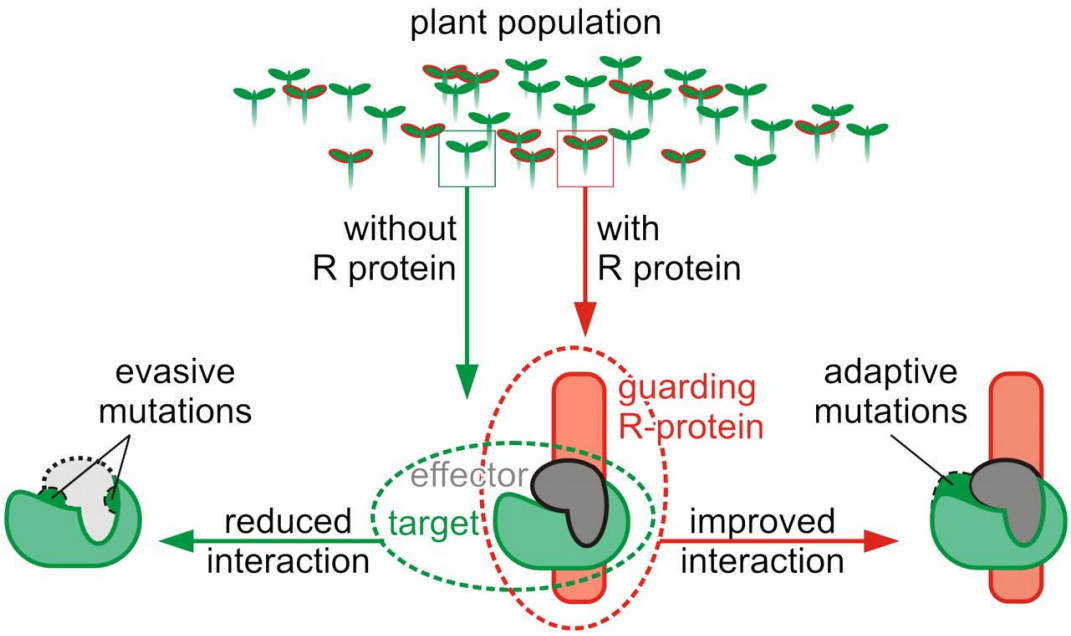

Figure I. 18 Opposing selection forces on guarded effector targets in a plant population polymorphic for R genes. In the absence of the R protein (green arrows), targets will be under selective pressure to reduce the interaction and evade manipulation (left). In the presence of the R protein (red arrows), the guarded effector target will be under selective pressure to improve the interaction with the effector and enhance pathogen perception (right). From: van der Horn et al., 2008

An example for the decoy model can be given with the tomato (*Solanum lycopersicum*) Ser/Thr kinase Pto, which interacts with the NB-LRR protein Prf and mediates recognition of the AvrPto and AvrPtoB proteins of *Pseudomonas syringae*. The actual virulence targets of AvrPto and AvrPtoB, however, appear to include the kinase domains of the receptor-like kinases CERK1, BAK1, EFR1 and FLS2, rather than the kinase domain of Pto (Shan et al., 2008; Gimenez-Ibanez et al. 2009). This could be interpreted as an indication that Pto is being used as a type of bait by Prf to interact with effectors proteins that normally target other kinases. In this context, Collier and Moffett (2009) proposed that the use of such recognition cofactors as bait is a common mechanism employed by NB-LRR proteins to sense Avr proteins and activate a molecular switch that results in the induction of resistance responses. In Figure I.19 the guard model classical, multiple and decoy model are contrasted.

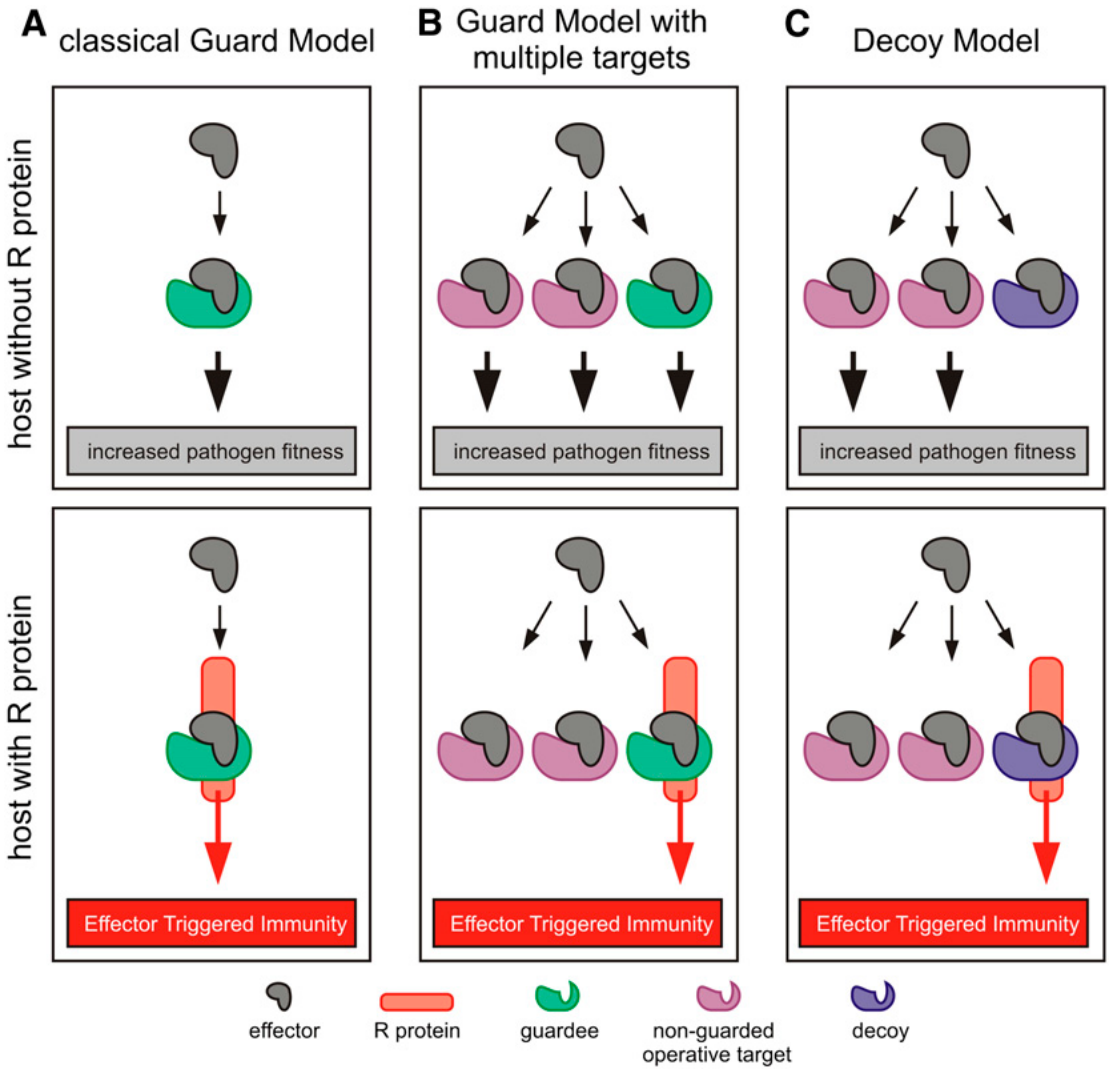

Figure I. 19 Comparisons of the Guard and Decoy Models. The classical Guard Model (A) is contrasted with a modified Guard Model in which the effector targets multiple plant proteins (B) and the Decoy Model (C). Effectors are depicted in gray, operative effector targets in purple, guardee in green, decoy in blue, and the R protein in orange (van der Horn et al., 2008).

3. Avirulence genes (effectors)

Avirulence gene is a gene encoding a protein which is specifically 'recognized' by genotypes of the host plant harbouring the matching resistance gene, regardless of its function or role in pathogenicity (Rouxel et al., 2010). Avirulence genes have positive roles in establishing infection, which the plant has evolved to recognize as an indicator of invasion (Catanzariti et al., 2007). This

concept was first developed in bacteria since they have both elicitor (avirulence) and virulence activities (van den Burg et al., 2003; Rooney et al., 2005) and after generalized to all plant pathogens (Rouxel et al., 2010).

Successful pathogens including viruses, bacteria, protozoans, fungi, oomycetes and nematodes use effectors to alter the plant's in morphology, physiology, biochemistry state to benefit colonization or to suppress the activation of host plant defenses (Bent and Mackey 2007; Torto-Alalibo et al. 2009).

Effector delivery occurs via diverse mechanisms. Physical injection via analogous systems is one mechanism by which diverse plant pathogens deliver effectors to their hosts. Plant parasitic nematodes possess a syringelike stylet for both effector delivery and nutrient uptake. The haustoria of certain biotrophic oomycetes and fungi are analogous structures formed when a hypha penetrates a host cell wall, causing host plasma membrane invagination.

There is no general common structure between avirulence gene products, except that most are secreted proteins. Because there would be no evolutionary advantage to a pathogen keeping a protein that only serves to have it recognized by the plant, it is believed that the products of *Avr* genes play an important role in virulence in genetically susceptible hosts. In fungi there is no proof that *Avr* proteins contribute directly to infection success. Although nothing is known about the function of these fungi *Avr* genes, all of them are subjected to diversifying selection, which implies that they are maintained in populations because they play a role in pathogenicity. Some of the *Avr* genes identified in rust fungi are upregulated within the haustoria, suggesting a role in the establishment of biotrophy (Catanzariti et al., 2007).

Of the vast number of bacterial effector proteins that have been cloned, only a few have been biochemically characterized. Characterized effectors possess enzyme activity and modify host proteins to promote bacterial virulence. Effectors may have evolved to target key components of PAMP-triggered defense, or they may target a variety of different host proteins to promote pathogenicity (Chisholm et al., 2006). Most cloned fungal effectors are small proteins of unknown function containing a signal for secretion into the apoplast (Chisholm et al., 2006). Most of these *Avr* genes show no sequence homology among each other.

4. Resistance Genes (R genes)

The resistance proteins provides plant two capabilities; firstly recognition of specific pathogen(s) and secondly activation of defense responses. Over 70 plant *R* genes controlling resistance against pathogens ranging from viruses, bacteria, fungi to nematodes have been isolated from different plant species (Liu et al., 2007; Xiao et al 2008;). (Table I.8). Among them several different classes of

R genes has been characterized. Comparative analysis of the cloned R- genes from different plants and providing resistance to different pathogens have revealed certain conserved amino acid domains that include nucleotide-binding sites (NBS), leucine-rich repeat (LRR), transmembrane domains (TM) and serine/threonine protein kinases (PK's) arranged in different combinations (Hammond-kosack and Jones 1997). Based on the arrangements of different domains found in R proteins they can be classified into few classes (Figure I.20). A few dominantly inherited R proteins do not fit these five major classes. These include maize *Hm1*, Arabidopsis *RPW8*, sugar beet *Hs1pro1*, tomato *Ve* proteins, barley *Rpg1*, Arabidopsis *RFO1*, rice *Xa21* and rice Pi-d2 (Hammond-Kosak and Kanuya, 2007).

The NBS-LRR genes represent the largest class of R genes (Martin et al, 2003) and encode proteins with a variable N-terminal domain of approximately 200 amino acids, connected by predicted NBS domain of approximately 300 aa and a more variable tandem array of approximately 10 to 40 short LRR motifs (Jones and Jones, 1997).

So far, among the cloned resistance genes, which confer resistance to fungal and bacterial pathogens only four, are recessive: barley *mlo*, Arabidopsis *edr1*, Arabidopsis *rrs1*-r and rice *xa5*. Whilst the functions of dominantly inherited R gene products are beginning to emerge, the molecular mechanisms involved in recessive resistance are less well understood (Hammond-Kosak and Kanuya, 2007).

Table I. 8 Non exhaustive list of identified disease resistance genes in plants (Liu et al, 2007)

Gene	Host	Pathogen	Protein type	Reference
RPS2	*Arabidopsis*	*Pseudomonas syringae*	CC-NBS-LRR	*Science*, 1994, 265: 1856-1860
RPS5	*Arabidopsis*	*Pseudomonas syringae*	LZ-NBS-LRR	*Plant Cell*, 1998, 10: 1439-1452
RPM1	*Arabidopsis*	*Pseudomonas syringae*	CC-NBS-LRR	*PNAS*, 1998, 95: 15849-15854
RPP8/HRT	*Arabidopsis*	*Peronospora parasiticia*	CC-NBS-LRR	*Plant Cell*, 1998, 10: 1861-1874
RPP13	*Arabidopsis*	*Peronospora parasiticia*	CC-NBS-LRR	*Plant J*, 2000, 21: 177-188
RCY1	*Arabidopsis*	*Cucumber mosaic virus*	CC-NBS-LRR	*Plant J*, 2002, 32: 655-667
RPP/HRT	*Arabidopsis*	*Turnip crinkle virus*	CC-NBS-LRR	*Plant Cell*, 2000, 12: 663-676
RPM1	Tomato	*Pseudomonas syringae*	CC-NBS-LRR	*Science*, 1995, 269: 843-846
Prf	Tomato	*Pseudomonas syringae*	CC-NBS-LRR	*Cell*, 1996, 86: 123-133
I2	Tomato	*Fusarium oxysporum*	CC-NBS-LRR	*Plant Cell*, 1997, 9: 521-532
Mi-1	Tomato	*Meloidogyne javanica*	CC-NBS-LRR	*Plant Cell*, 1998, 10: 1307-1319
Mi-9	Tomato	*Meloidogyne javanica*	CC-NBS-LRR	*Plant Physiol*, 2007, 143: 1044-1054
Sw-5/Mi	Tomato	*Tospovirus*	CC-NBS-LRR	*Mol Plant-Microbe Interact*, 2000, 13: 1130-1138
Rx2	Potato	*PVX* (Potato X virus)	CC-NBS-LRR	*Plant Cell*, 1999, 11: 781-792
Gpa2/Rx1	Potato	*Globodera pallida/PVX*	CC-NBS-LRR	*Plant J*, 2000, 23: 567-576
R1	Potato	*Phutophthora infestans*	CC-NBS-LRR	*Plant J*, 2002, 30: 361-371
Mla1	Barely	*Blumeria graminis*	CC-NBS-LRR	*Plant Cell*, 2001, 13: 337-350
Mla6	Barely	*Blumeria graminis*	CC-NBS-LRR	*Plant J*, 2001, 25: 335-348
Mla12	Barely	*Blumeria graminis*	CC-NBS-LRR	*Plant Cell*, 2003, 15: 732-744

Mla13	Barely	*Blumeria graminis*	CC-NBS-LRR	*Plant J*, 2004, 38: 215-226
Pib	Rice	*Magnaporthe grisea*	CC-NBS-LRR	*Plant J*, 1999, 19: 55-64
Pi-ta	Rice	*Magnaporthe grisea*	CC-NBS-LRR	*Plant Cell*, 2000,12: 2033-2046
Pi36	Rice	*Magnaporthe grisea*	CC-NBS-LRR	*Genetics*, 2007: doi:10.1534
Xa1	Rice	*Xanthomona oryzae*	CC-NBS-LRR	*PNAS*, 1998, 95: 1663-1668
Rp1	Maize	*Puccinia sorghi*	CC-NBS-LRR	*Plant Cell*, 1999,11: 1365-1376
Dm3	Lettuce	*Bremia lactucae*	CC-NBS-LRR	*Plant Cell*, 1998,10: 1818-1832
Bs2	Pepper	*Xanthomonas campestris*	CC-NBS-LRR	*PNAS*, 1999, 96: 14153-14158
Pm3b	Wheat	*Blumeria graminis* f. sp.*Tritici*	CC-NBS-LRR	*Plant J*, 2004, 37: 528-538
Lr10	Wheat	*Puccinia triticina*	CC-NBS-LRR	*PNAS*, 2003, 10: 15253-15258
Pl8	Sunflower	*Plasmopara halstedii*	CC-NBS-LRR	*J Experi Botany*, 2005, 56: 567-575
RPS4	*Arabidopsis*	*Pseudomonas syringae*	TIR-NBS-LRR	*Plant J*, 1999, 20: 265-277
RPP1	*Arabidopsis*	*Peronospora parasiticia*	TIR-NBS-LRR	*Plant Cell*, 1998, 10: 1847-1860
Rpp10	*Arabidopsis*	*Peronospora parasiticia*	TIR-NBS-LRR	*Plant Cell*, 1998, 10: 1847-1860
Rpp14	*Arabidopsis*	*Peronospora parasiticia*	TIR-NBS-LRR	*Plant Cell*, 1998, 10: 1847-1860
RPP4	*Arabidopsis*	*Peronospora parasiticia*	TIR-NBS-LRR	*Plant J*, 2002, 29: 439-451
RPP5	*Arabidopsis*	*Peronospora parasiticia*	TIR-NBS-LRR	*Plant Cell*, 1997, 9: 879-894
SSI4	*Arabidopsis*	*P. syringae* pv. *maculicola*	TIR-NBS-LRR	*Plant Cell*, 2002, 14: 3149-3162
L6	Flax	*Melampsora lini*	TIR-NBS-LRR	*Plant Cell*, 1995, 7: 1195-1206
L, L1-L11, LH	Flax	*Melampsora lin*	TIR-NBS-LRR	*Plant Cell*, 1999, 11: 495-506
M	Flax	*Melampsora lini*	TIR-NBS-LRR	*Plant Cell*, 1997, 9: 641-951
P	Flax	*Melampsora lini*	TIR-NBS-LRR	*Plant Cell*, 2000, 13: 163-178
P2	Flax	*Melampsora lini*	TIR-NBS-LRR	*Plant Cell*, 2002, 13: 163-178
Bs4	Tomato	*Xanthomonas campestris*	TIR-NBS-LRR	*Plant J*, 2004, 37: 46-60
N	Tobacco	*T*obacco *m*osaic *v*irus	TIR-NBS-LRR	*Cell*, 1994, 78: 1011-1015
RRS1-R	*Arabidopsis*	*Ralstonia solanscearum*	WRKY-TIR- NBS-LRR	*PNAS*, 2002, 99: 2404-2409
Pi9	Rice	*Magnaporthe grisea*	NBS-LRR	*Genetics*, 2006, 172: 1901-1914
Pi2	Rice	*Magnaporthe grisea*	NBS-LRR	*Mol Plant-Microbe Interact*, 2006, 11: 1216-1228
Piz-t	Rice	*Magnaporthe grisea*	NBS-LRR	*Mol Plant-Microbe Interact*, 2006, 11: 1216-1228
Cre3	Wheat	*Heterodera avenuae*	NBS-LRR	*Genome*, 1997, 40: 659-665
Cre1	Wheat	*Heterodera avenuae*	NBS-LRR	*Mol Plant-Microbe Interact*, 2003, 16: 1129-1134
I2C	Tomato	*Fusarium oxysporum*	NBS-LRR	*Plant Cell*, 1997,9:521-532
Hero	Tomato	*Globodera rostochiensis*	NBS-LRR	*Plant J*, 2002,31:127-136
Rpg1	Barley	*Puccinia graminis*	Protein kinase	*PNAS*, 2002, 99: 9328-9333
RPP27	*Arabidopsis*	*Peronospora parasiticia*	Receptor like protein	*Plant Physiol*, 2004, 135: 1100-1112
Pto	Tomato	*Pseudomonas syringae*	Ser/Thr protein kinase	*Science*, 1993,262: 1432-1436
Xa21	Rice	*Xanthomona oryzae*	LRR-Ser/Thr protein kinase	*Science*, 1995, 270: 1804-1806
Xa21D	Rice	*Xanthomona oryza*	LRR receptor- like protein	*Plant Cell*, 1998, 10: 765-780
Xa26	Rice	*Xanthomona oryzae*	LRR receptor- like kinase	*Plant J*, 2004, 37: 517-527
RFO1	*Arabidopsis*	*Fusarium oxysporum*	Receptor-like kinase	*Genetics*, 2005, 171:305-321
Pi-d2	Rice	*Magnaporthe grisea*	B-lectin PK	*Plant J*, 2006, 46: 794-804
Cf-9	Tomato	*Cladosporium fulvun*	LRR-TM	*Science*, 1994, 266: 789-793

Cf-2	Tomato	*Cladosporium fulvun*	LRR-TM	*Cell*, 1996, 84: 451-459
Cf-4	Tomato	*Cladosporium fulvun*	LRR-TM	*Plant Cell*, 1997, 9: 2209-2224
Hcr9-4E	Tomato	*Cladosporium fulvum*	LRR-TM	*Mol Microbiol*, 2004, 54: 533-545
Cf-2/5	Tomato	*Cladosporium fulvun*	LRR-TM	*Plant Cell*, 1998, 10: 1915-1925
Ve	Tomato	*Verticillium dahliae*	LRR-TM	*PNAS*, 2001, 98: 6511-6515
Hsl^{pro-1}	Beet	*Heterodera schachtii*	LRR-TM	*Science*, 1997, 275: 832-834
Rpw8	*Arabidopsis*	*Erysiphe cruciferarum*	TM-CC	*Science*, 2001, 291:118-120
Mlo	Barely	*Erysiphe graminis*	TM	*Cell*, 1997, 88: 695-705
Xa5	Rice	*Xanthomona oryzae*	TF II A Trans-cription factor	*Mol Genet Genomics*, 2006, 275: 354- 366
Xa27	Rice	*Xanthomona oryzae*	No homolog	*Nature*, 2005, 435:1122-1125
Xa13	Rice	*Xanthomona oryzae*	Homolog of nodulin MtN3	*Genes Dev*, 2006, 20: 1250-1255

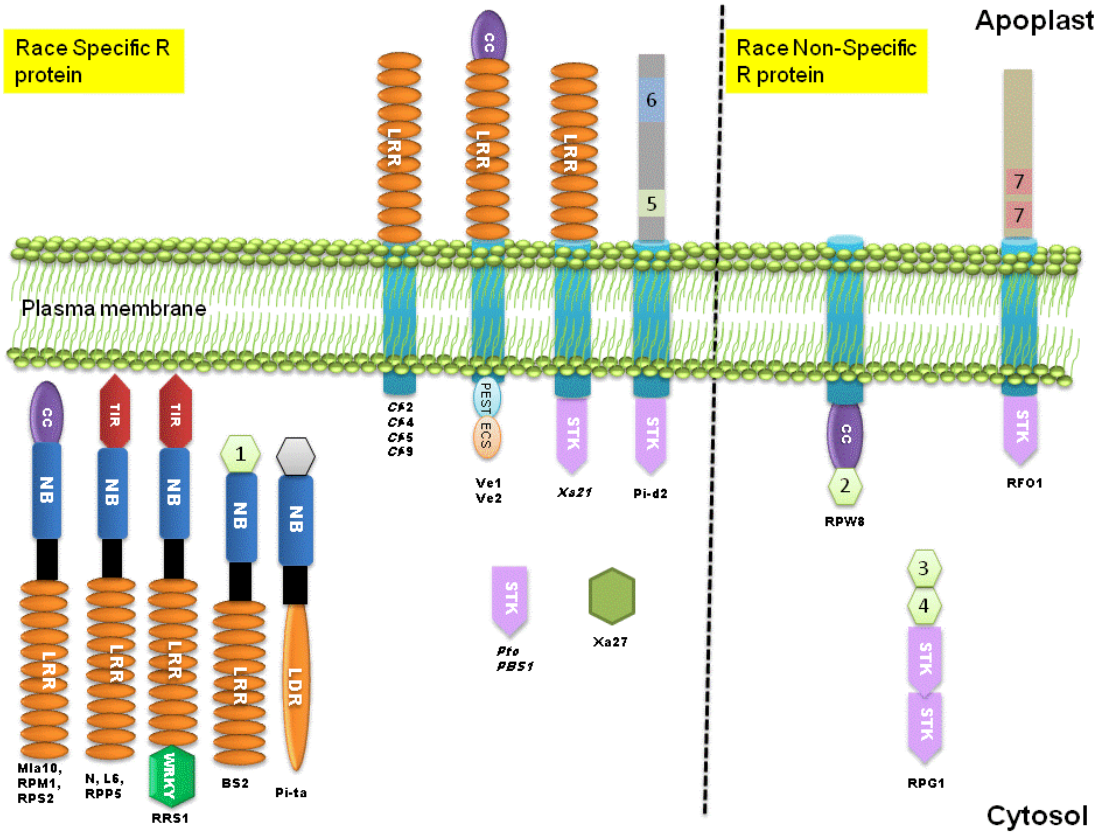

Figure I. 20 Classes of R protein and their cellular location. CC, coiled-coil domain; TIR, Toll and Interleukin 1 receptor-like motif; NB, nucleotide binding site; LRD, leucine-rich domain; LRR, leucine-rich repeat; NLS, nuclear localization signal; ECS, endocytosis signal; PEST, Pro-Glu-Ser-Ther-like sequence; STK serine threonine kinase, WRKY, 1, 2, 3, 4 novel domains that lack significant homology to known proteins; 5, domain with homology to a B-lectin; 6, structure with a weak similarity to a PAN domain; 7, structure with homology to epidermal growth factor (EGF)-like domain. Adptated from: Hammond-Kosack & Kostya Kanyuka, 2007.

4.1 Classes of Resistance Gene

4.1.1 RLP and RLK class of R genes

R proteins including class that comprises cell surface receptor-like transmembrane proteins (RLP) and receptor-like kinases (RLK). The common feature of these proteins is that they possess an extracellular LRR (eLRR) domain. Representatives of RLP R proteins are tomato Cf proteins conferring resistance to the tomato fungal pathogen *Cladosporium fulvum* (Jones et al. 1994; Hammond- Kosack and Jones 1997) and *Arabidopsis RPP27* conferring resistance to the oomycete *Hyaloperonospora parasitica* (Tor et al. 2004). RLK R proteins are represented by rice Xa21 and Xa26, both of which confer resistance to multiple strains of *Xanthomonas oryzae* pv. *oryzae* (Song et al. 1995; Sun et al. 2004).

The remaining *R* genes encode proteins that either resembles the overall structure or a domain of the above two classes with some degree of structural variation, or have a novel protein structure that does not show significant homology to any other R proteins. Therefore, they are atypical *R* genes in comparison with the LRR-encoding *R* genes. For example, tomato *Pto* and *Arabidopsis PBS1* encode members of a conserved protein kinase family (Martin et al. 1993; Swiderski and Innes 2001) that resemble the cytoplasmic protein kinase domain of RLK R proteins.

4.2 The NBS-LRR class

Nucleotide Binding Site-Leucine Rich Repeats (NBS-LRR) proteins are some of the largest proteins known in plants, ranging from about 860 to about 1,900 amino acids (McHale et al., 2006). They have at least four distinct domains joined by linker regions: a variable amino-terminal domain, the NBS domain, the LRR region, and variable carboxy-terminal domain (Hammond-Kosack and Jones 1997; Dangl and Jones 2001, McHale et al., 2006). The NBS of intracellular plant R proteins and animal Nod1/Nod2 proteins, which function in inflammatory and immune responses (Inohara et al., 2005), is part of a larger domain called NB-ARC, because it is shared between R proteins of both human apoptotic protease-activating factor 1 (APAF-1) and its *Caenorhabditis elegans* homolog CED-4 (Van der Biezen and Jones 1998).

The ARC domain can be further subdivided into two structurally and functionally distinct units; ARC1 and ARC2, where the ARC1 motif is required for the binding between the CC-NB and the LRR of the R protein, and the ARC2 motif is required for switching on/off the R molecule through its interaction with the LRR domain (Rairdan and Moffett 2006). Together, the NB and ARC domains

comprise a functional nucleotide binding pocket. These R proteins confer resistance to various pathogens and can be further subdivided into two groups, based on their N-terminal features. The first group contains N-terminal domains that resemble the cytoplasmic signaling domain of the *Drosophila toll* and human interleukin-1 receptors (TIR) and are called TNL (TIR-NBS-LRR) (Whitham et al. 1994; Lawrence et al. 1995). The second group contains (in most cases) a coiled coil (CC) domain and is often referred to as CNL (CC-NBS-LRRs) (Bent et al. 1994; Grant et al. 1995).

Proteins carrying an NB–ARC domain belong to the STAND (Signal Transduction ATPases with Numerous Domains) family of NTPases (Leipe et al., 2004) and can be differentiated by many conserved the NB–ARC domain, such as a central domain nucleotide-binding site, kinase-1a or P-loop, kinase 2, and kinase 3a, and it may affect R protein function through nucleotide binding, hydrolysis, and the control of cell death (Martin et al. 2003; Lukasik and Takken, 2009). The schematic representation of a typical NB–LRR protein is shown in Figure I.21.

The NB domains of TNLs and CNLs sub class are distinguished by the sequences of three motifs: RNBS-A, RNBS-C, and RNBS-D present in NB domain (Meyers et al., 2003). TNL group could also have a WRKY domain attached to the LRR at the C-terminus as is the case of *Arabidopsis* RRS1-R protein for example (Deslandes et al. 2002).

The CC domain is sometimes joined and/ or replaced by a Solanaceae domain (SD) or a predicted BED DNA binding domain. Alternatively, a number of predicted NB-LRR proteins of both the TNLs and CNLs (as defined by the configuration of their NB-ARC domains) seem to possess no N-terminal sequence in their NB domain (Absent). The N-termini of CNL proteins show little sequence or structural similarity. Even within the loosely defined CC domains the only apparently widely conserved feature is a small ‘‘EDVID’’ motif, which defines the largest subclass of CC domain (Collier and Moffet, 2009).

Both TNLs and CNLs include members that experience alternative splicing. The induction of splice variants upon pathogen recognition has been observed for plant NB-LRR proteins, suggesting that alternative splicing may have a regulatory role in the plant defense response (Jordan et al., 2002).

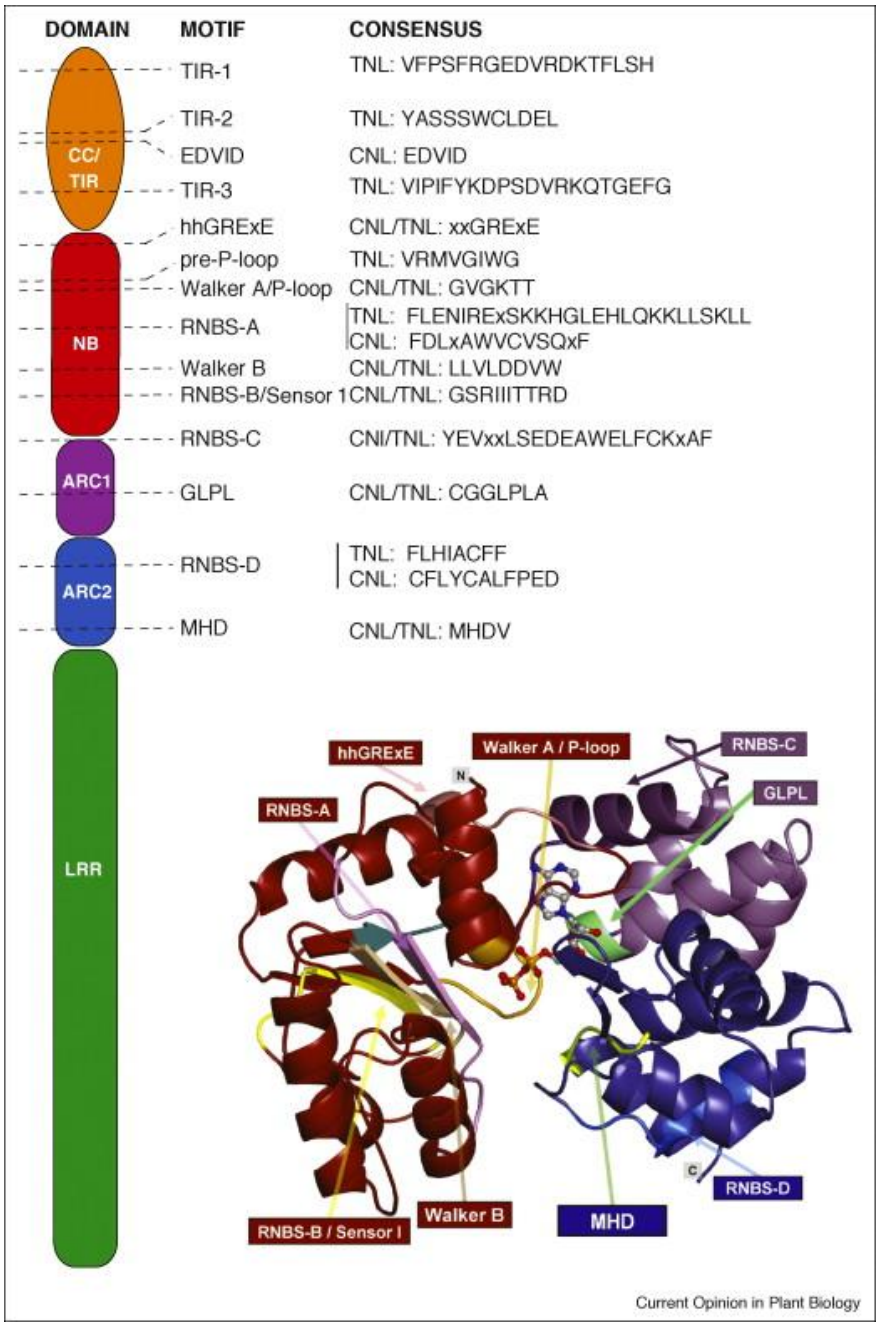

Figure I. 21 Schematic representation of a typical NB–LRR protein. The (sub) domains are depicted as coloured boxes: CC/TIR domain (orange), NB (red), ARC1 (purple), ARC2 (blue) subdomains and LRR domain (green) whereas conserved motifs are marked as lines. Consensus of sequences is written next to name of the motif. Insert, predicted 3D structure of the NB–ARC domain of I-2 modelled on the ADP-bound Apaf1 (1z6t) template Conserved motifs and N- and C-termini are marked. ADP and Mg atoms are depicted as balls and sticks (Lukasik and Takken 2009).

4.2.1 The function of main domains in NB-LRR proteins

4.2.1.1 Nucleotide binding site (NBS)

The NBS domain works binding ATP, ADP which initiates conformational changes in plant NBS-LRR proteins, resulting in its activation (Leipe et al., 2004; Young & Innes, 2006).

The crystal structures of the NBS domains of mammalian apoptotic protease activating factor 1 (Apaf-1) and the *Caenorhabditis elegans* cell death protein CED-4 has provided informative insights into the spacial arrangement and function of the conserved motives in NBS plant domains. The NBS domain of Apaf-1 is composed of four subdomains: a three-layered α-β domain (which contains the Ploop), a helical domain, an extended winged-helix domain (containing the MHD motif) and a second helical domain (also called ARC1, ARC2 and ARC3). The structure of CED-4 is similar to that of Apaf-1, but it lacks ARC3. Sequence analysis suggests that plant NBS-LRR proteins have a domain structure similar to that of Apaf-1 and CED-4, but like CED-4 they lack ARC3. The crystal structures of both CED-4 and Apaf-1 indicate that an ATP or ADP is bound in a pocket that is mostly confined in the NBS domain (Young & Innes, 2006).

The NBS domain is highly conserved among R proteins (McHale et al., 2006). The presence of conserved motifs such as a central domain nucleotide-binding site, kinase-1a or P-loop, kinase 2, and kinase 3a may affect R protein function through nucleotide binding, hydrolysis, and the control of cell death (Martin et al., 2003).

The conserved motif within the NB domain has been used extensively to identify resistance genes homologs in model and crop species (Kanazin et al., 1996; Aarts et al., 1998; Penuela et al., 2002; Zhu et al., 2002; Ferrier-Cana et al., 2003; Yaish et al., 2004; Noir et al., 2004; Palomino et al., 2006).

4.2.1.2 LRR (Leucine-rich repeats)

The LRR domain, firstly recognized in the leucine-rich 2-glycoprotein, correspond to tandemly repeats arrayed in the N-terminus of R-genes and is involved in primarily specific recognition of pathogen effectors (Jones and Jones, 1997; Hammond-Kosack and Jones 1997; Ellis et al. 2000; Van der Hoorn et al. 2001). All LRRs motifs are divided into a highly conserved part and a variable part. The highly conserved part consists of an 11-residue stretch, LxxLxLxxNxL, or a 12-residue stretch, LxxLxLxxCxxL, in which "L" is any aliphatic amino acid (more frequently Val, Leu, or Ile), but also phenylalanine, methionine and alanine, "N" is Asn occasionally replaced by a cysteine

or threonine, and some repeats contain a glutamine receptor or a serine at this position and x is other residues distributed in a non-random fashion among the hydrophobic amino acid (Kobe and Deisenhofer, 1994; Buchanan and Gay 1996). These aminoacid distribution forms a β-sheet (Buchanan and Gay1996; Jones and Jones, 1997; Kajava, 1998; McHale et al. 2006). Usually, the LRR motif contains 20-30 residues (Kobe and Kajava, 2001; Bella et al., 2008) but up to 47 duplicated LRRs have been found (Kuang et al., 2004).

The 3D structural arrangement of LRRs was first achieved in the crystal structure of ribonuclease inhibitor (RI). The structure of porcine RI showed that LRRs corresponded to structural units, each consisting of a β-strand and α-helix connected by loops (Kobe and Deisenhofer, 1993) that form the concave face shaped like a horseshoe or banana.

LRR proteins can be subdivided into at least seven subfamilies, characterized by different lengths and consensus sequences of the variable parts of the repeats (Ohyangi &Matsushima, 1997; Kajava 1998). In this classification repeats from different subfamilies never occur together in the same protein and probably evolved independently (Kobe and Kajava, 2001; Bella et al., 2008) (Table I.9).

R-proteins from plants share LRRs of the extracellular or extracytoplasmic type, characterized by the consensus sequence LxxLxxLxLxxNxLT/SGxIPxxLGx (Kajava, 1998).

The crystal structure of the isoform 2 of polygalacturonase-inhibiting proteins (PGIPs) from *Phaseolus vulgaris* (PvPGIP2) is the only available structure of plant LRR proteins (Di Matteo et al., 2003). Many plants produce extracellular PGIPs that specifically recognize and inhibit fungal and insect endo-polygalacturonase (PG) enzymes that are responsible for degrade the polysaccharides of the cell wall. Thus, LRRs of the extracellular type homologous to PGIP are found in pattern-recognition receptors involved in non-host specific defense.

Table I. 9 Sub-families of LRR proteins (Adapted from: Kobe and Kajava, 2001)

LRR subfamily typical	LRR length (range)	Organism origin	Cellular location	Structures available
RI-like	28–29 (28–29)	Animals	Intracellular	RI, rna1p
SDS22-like,	22 (21–23)	Animals, fungi	Intracellular	U2A', TAP, RabGGT, LC1, InlB
Cysteine- containing	26 (25–27)	Animals, plants, fungi	Intracellular	Skp2
Bacterial	20 (20–22)	Bacteria Gram-negative	Extracellular	YopM
Typical	24 (20–27)	Animals, fungi	Extracellular	No
Plant-specific	24 (23–25)	Plants, primary eukaryotes	Extracellular	PvPGIP2
TpLRR	23 (23–25)	Bacteria	Extracellular	No

The PvPGIP2 fold consists of a central LRR domain flanked by the N- and C-terminal cysteine-rich regions (Figure I.22 b) (Di Mateo et al., 2006; Bent and Mackey, 2007). The LRR domain is characterized by the tandem repetition of 10 coils matching the consensus sequence xxLxLxxNxLt/sGxIPxxLxxLxxL (Figure I.22 a). An extended parallel β-sheet occupies the concave inner side of the protein solenoid (Concave face) (Figure I.22 c). It is in the β-sheet where the residues determining the affinity and the specificity of PGIP2 reside. Nine 310-helices are located on the convex side of the protein (Fig I.22 c). Specific positions of the LRR repeat (red circles in figure c) are occupied by hydrophobic amino acids, mostly leucines that point into the interior of the protein scaffold and stabilize the overall fold topology thorough van der Waals interactions. Position 8 (Figure I.22 c) is usually occupied by asparagine residues that are oriented towards the core of the protein and form hydrogen bonds with the carbonyls main chain or amide groups originating the typical ‘‘asparagine ladder’’. The convex face of the LRR region of PGIP2 is mostly occupied by unstructured segments that are stabilized through water molecules; these are organized in spines along the structure and form H-bond interactions with the protein backbone. While contributing to stabilize the protein scaffold, the water network provides a structural flexibility to the molecule and might facilitate the adaptation of the PGIP scaffold to the surface of its interacting partners. The N-terminal region contains two disulphide bridges and consists of a 15 residue long α-helix and a short β-strand that forms H-bonds with residues in convex face. The C-terminal region contains two disulphide bonds and consists of the last two α-helices. The LRR-flanking regions play a structural role in capping the hydrophobic core of the protein. The β-strand/β-turn region is primary candidate for determining pathogen specificity (Figure I.22 d).

Within the tomato *Cf* gene family, the comparative analysis of *Cf-4* and *Cf-9*, which confer resistance to *C. fulvum* through recognition of different avirulence determinants, has shown that 33 out of the 57 amino acids which distinguish the two proteins are located within the interstitial amino acid residues of the β-sheet/β-turn region (Thomas *et al.*, 1997).

In many NB-LRR genes, analyses of corresponding proteins have found elevated ratios of nonsynonymous to synonymous substitutions in the leucine-rich (LRR) domain, mainly in the putative solvent exposed residues (i.e. the x residues), indicating that the LRR domain is subject to positive selection for amino acid diversification and consequently for pathogen specificity (Parniske et al. 1997; Meyers et al., 1998; Botella et al., 1998; Michelmore and Meyers 1998; McDowell et al. 1998; Ellis et al., 1999; 2000; Dodds et al. 2001; Shen et al., 2003; Yahiaoui et al., 2006; Geffroy et al., 2009). Moreover single substitutions in the xxLxLxx motif can disturbed the function of R genes (Dixon *et al.*, 1998). A glutamate to lysine substitution in the xxLxLxx of the third LRR in the *Arabidopsis RPS5* gene product generates a protein which partially compromises the function of several *R* genes (Warren *et al.*, 1998). Single amino acid changes in *RPS2* and *RPM1* resistance genes of *Arabidopsis* abolished the ability to confer resistance to *P. syrangae* (Bent *et al.*, 1994; Mindrinos

et al., 1994; Grant *et al.*, 1995). Other examples are the proteins *Xa21* of rice and *M* of flax, where alterations of the LRR domains appear to be crucial for recognition specificity (Anderson *et al.*, 1997; Ronald, 1997).

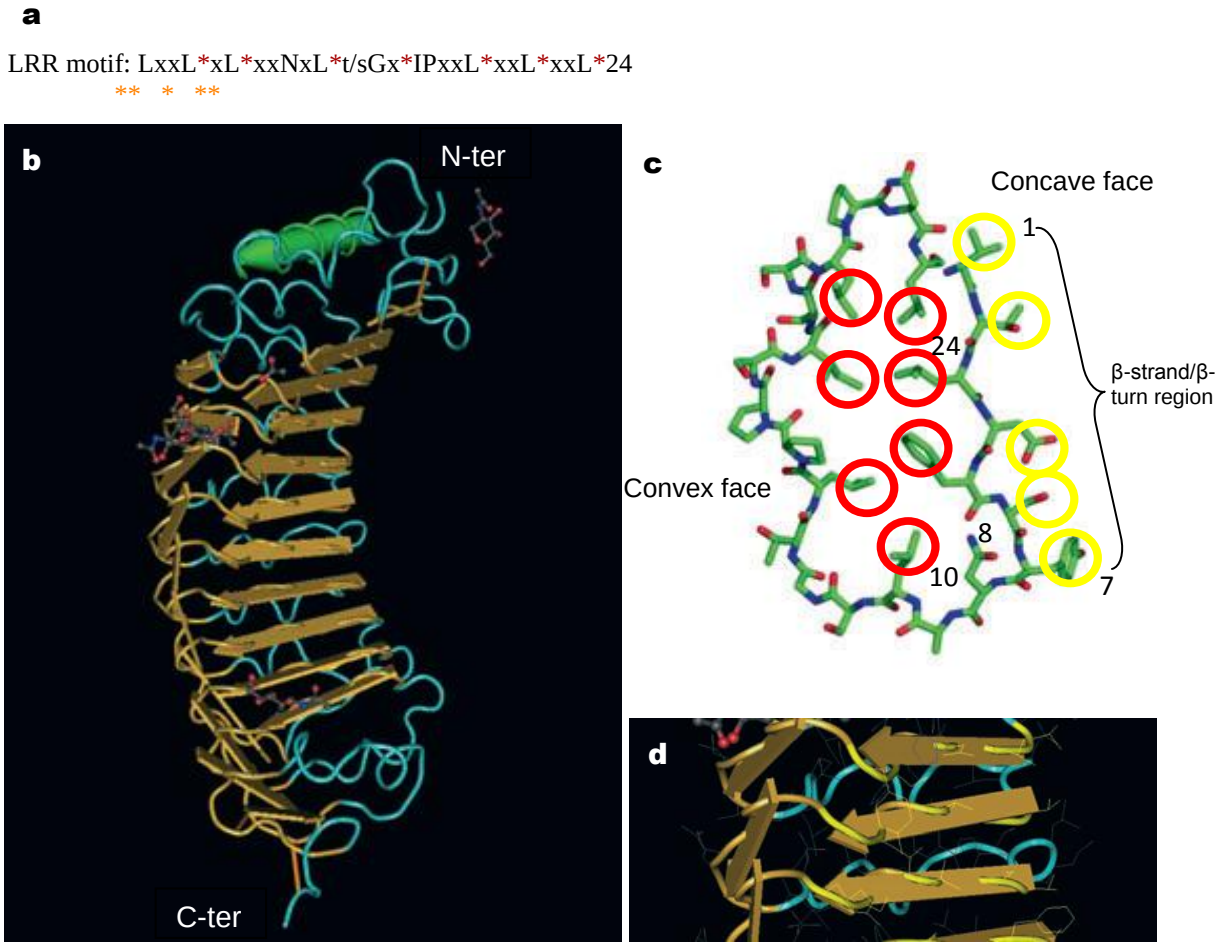

Figure I. 22 Leucine-rich repeat (LRR) structure of the PvPGIP2. (*a*) Consensus amino-acid motif for a plant extracellular LRR. (*b*) An LRR protein; polygalacturonase inhibiting protein of *Phaseolus vulgaris*. A typical LRR domain carries 21–25 amino acids per repeat and forms a large helix of multiple such repeats. The entire LRR domain is curved and the concave surface carries a β-sheet (β-strand/β-turn region, *yellow* and *gold* highlighting). (*c*) A single LRR (transverse section through the structure in *b*). The leucines and other hydrophobic residues that occur at regular intervals are driven to the protein interior in an aqueous environment (*red* highlight in *c*; *asterisks* in *a*), leaving the more variable "x" residues exposed on the protein surface. (*d*) Close-up of the β-strand/β-turn region, where 5 solvent exposed residues per repeat (*yellow* highlighted in *a*, *c*, and *d*) are primary candidates for determining pathogen specificity. From: Bent and Mackey, 2007.

4.1.2.3 TIR domain

The TIR domain was originally characterized due to homology with the intracellular regions of the mammalian IL-1 receptor (IL-1R) and the *Drosophilla* protein Toll, which contains 3 "boxes" of conserved residues, set in a central sequence ranging from 135 to 160 aa and two interfaces mediating TIR domain interactions. TIR domain interactions between receptors and adaptors play a critical role in activating conserved cellular signal transduction pathways in response to bacterial lipopolysaccharide (LPS), microbial and viral pathogens, cytokines and growth factors (Xu et al., 2000).

Although most studies have demonstrated that R gene specificity resides largely in the LRR region, there are indications for the L class of flax rust resistance genes, that polymorphism in the TIR domain of the flax TNL protein L6 also affects the specificity of pathogen recognition (Ellis et al., 1999; Luck et al., 2000). Other example comes from tobacco TNL protein N that confers resistance to Tobacco Mosaic Virus (TMV). In this case the TIR domain was necessary and sufficient for association with the p50 Avr elicitor from TMV suggesting that the TIR domain acts as an adaptor between the pathogen Avr protein and the signaling function of the R protein (Burch-Smith et al., 2007).

4.1.2.4 Coiled Coil (CC) domain

The CC (also called LZ –leucine zipper) serves as oligomerization domain that means it mediates the subunit assembly of a large number of proteins (including structural proteins, motor proteins and transcription factors) by increasing structure stabilization and combined functions of different domains (Engel & Kammerer, 2000). The CC structure is conserved from viruses to plants and mammals and it has been predicted that approximately 5% of proteins encoded in sequenced genomes contain CCs. (Nooren et al. 1999).

Coiled coils usually contain a repeated pattern, *hpphppp*, of hydrophobic (*h*) and polar (*p*) amino-acid residues, referred to as a heptad repeat. The positions in the heptad repeat are usually labeled *abcdefg*, where *a* and *d* are the hydrophobic positions, often being occupied by isoleucine, leucine or valine (Lupas, 1996). Folding a sequence with this repeating pattern into an alpha-helical secondary structure causes the hydrophobic residues to be presented as a 'stripe' that coils gently around the helix in left-handed fashion, forming an amphipathic structure (Nooren et al. 1999).

A CC motif is common but not always present in the 175 amino acids amino-terminal of CNLs (Meyers et al., 2003). Some CNLs have large amino-terminal domains; tomato Prf, for example, has 1,117 amino acids amino-terminal of the NBS, much of which is unique to this protein (McHale et al., 2006).

5. How NBS-LRR proteins recognize pathogens and activate defense responses?

The bait and switch model elaborated by Collier and Moffett (2009) was proposed to explain how NB-LRR proteins translate pathogen recognition into defense activation. This model proposes a two-step recognition process involving interactions with both cellular cofactors (bait) and the LRR domain, which in turn activates the molecular switch leading to disease resistance (Figure I.23).

The bait and switch model states that "bait" cofactors play an integral role in preparing NB-LRR proteins to be functional while retaining the molecular switch in an inactive conformation until a structural change triggers release of auto-inhibition. This structural change might take multiple forms, such as modification, removal, or conformational change of the bait protein or simply complex formation with the bait protein. At the same time, at least some bait proteins, such as NRIP1 and WRKY proteins, seem to interact with their cognate NB-LRR proteins only in the presence of the Avr protein. In these cases, the preparing of NB-LRR proteins might occur independently of bait proteins, or *via* interaction with bait protein might occur concomitantly with alterations that cause activation. Alterations of bait proteins could be perceived and relayed to the molecular switch via the N-terminal domain. Likewise, through the proximity induced by interdomain interactions, some recognition cofactors might bring multiple Avr proteins directly into contact with the autoinhibitory surfaces of the LRR/ARC2 interface. This might reinforce relatively weak, but specific, interactions between Avr and LRR domains which would in turn disrupt the autoinhibitory effect of specific LRR/ARC2 combinations.

The model can be summarized as follow: (a) The NB-LRR protein is "prepared" but in autoinhibited state – the ''switch'' is in the ''off'' position. This state requires a functional nucleotide binding pocket (presumably bound to ATP or ADP) and multiple intramolecular interactions including a fine-tuned balance between the LRR and ARC2. The recognition of cofactor bait might also be a critical component of the primed state. (b) The pathogen Avr protein is brought into the NB-LRR system *via* the bait protein, either through direct binding or through alteration to the bait. (c) The switch is changed to "on" position. The presence of the Avr protein causes perturbation and

subsequent conformational changes to the LRR/ARC2 interface, ultimately resulting in signal initiation. Conformational changes within the nucleotide binding pocket allow signaling motif(s) within the NB domain (green star) to associate with downstream signaling components. (d) Concurrent with or subsequent to signaling, intramolecular interactions within the NB-LRR protein are probably dissociate and reset, possibly allowing the protein to undergo repeated rounds of recognition, signaling and resetting given the continued presence of the Avr protein (Figure I.23).

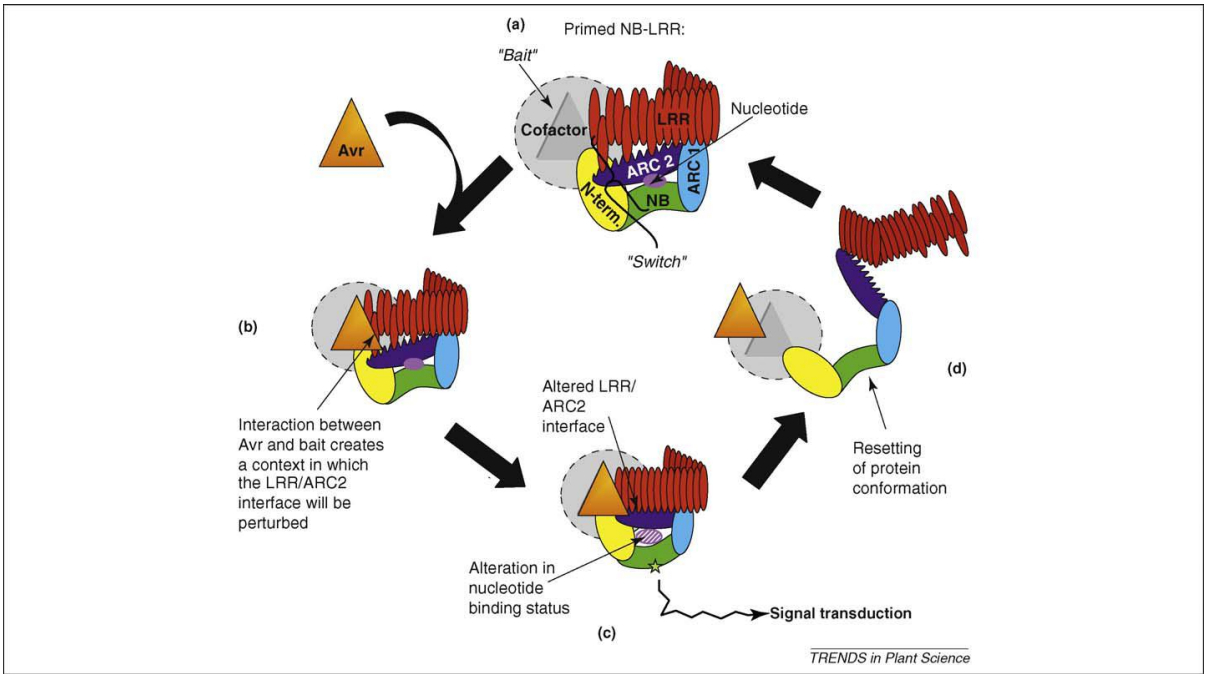

Figure I. 23 Bait and switch model of NB-LRR protein function. From: Collier and Moffett (2009).

6. Expression of R proteins

Recently, multiple analytical approaches such as expressed sequenced tag (EST), massively parallel signature sequencing (MPSS), microarray analysis and rapid amplification of cDNA ends (RACE) PCR have been used for analyzing the level, tissue specificity and possible inducibility of expression for approximately 170 NB-LRR-encoding and related genes in *Arabidopsis thaliana* (Tan et al., 2007). These analysis revealed that transcripts of most NBS-LRR-encoding and related genes are present at low levels in unchallenged plants and many showed tissue specific expression patterns. The transcript levels of the majority of NB-LRR-encoding and related genes are not altered during the plant defense response or by treatments with plant defense signaling molecules such as (salicylic acid)

SA or (jasmonate) JA. However, for some genes the expression was altered by SA and may be indicative of altered levels of surveillance by the plant (Tan et al., 2007). These results are in agreement with previous small-scale experiments (Navarro et al., 2004; Zipfel et al., 2004).

7. Plant-pathogen co evolution

In the plant-pathogen battle, if an R protein detects an Avr protein and triggers host resistance selection pressure should be imposed on the pathogen carrying the *Avr* gene to evade recognition. This could be achieved by structural changes in the Avr protein through mutations without necessarily affecting its virulence function. Once the host resistance is overcome, the selection pressure would be on the plant to generate a new *R* gene to recognize the mutated *Avr* gene (Xiao et al., 2008).
Pathogens reproduce much faster than plants, thus plant must somehow compensate the long generation. This compensation could be represented by the elevate number of R genes maintained in the plant genomes and by structural features of R proteins. Whereas Avr proteins are structurally diverse, presumably performing very different virulence functions in the host, most R proteins possess a highly conserved NB domain along with hyper-variable LRR. This means a new Avr allele that evades R recognition may only evolve from the existing alleles through mutations or allelic recombination while R genes in a cluster of highly homolog could evolve through diverse mechanism including: point mutations, intra-and inter-genic recombinations, gene conversions and unequal crossing over (Xiao et al., 2008). A model for plant pathogen co evolution is shown in Figure I.24

7.1 Evolution of R genes at plant population level

Two models have been proposed to explain R genes evolution. The **birth-and-death** model or "arm race", assumes that R genes emerge in a plant population (birth) by sequence recombination, point mutation and are lost when the resistance in that plant population is broken (death), thus new R genes should be continuously generated to ensure adequate plant protection (Michelmore & Meyers 1998). Support for these evolutionary dynamics centers on the common observation that amino acids evolve at a faster rate in functionally important regions of R-gene proteins than the corresponding rate of synonymous changes (Parniske et al., 1997; Botella et al., 1998; McDowell et al., 1998; Meyers et al., 1998, Ellis et al., 1999; Rose et al., 2004).

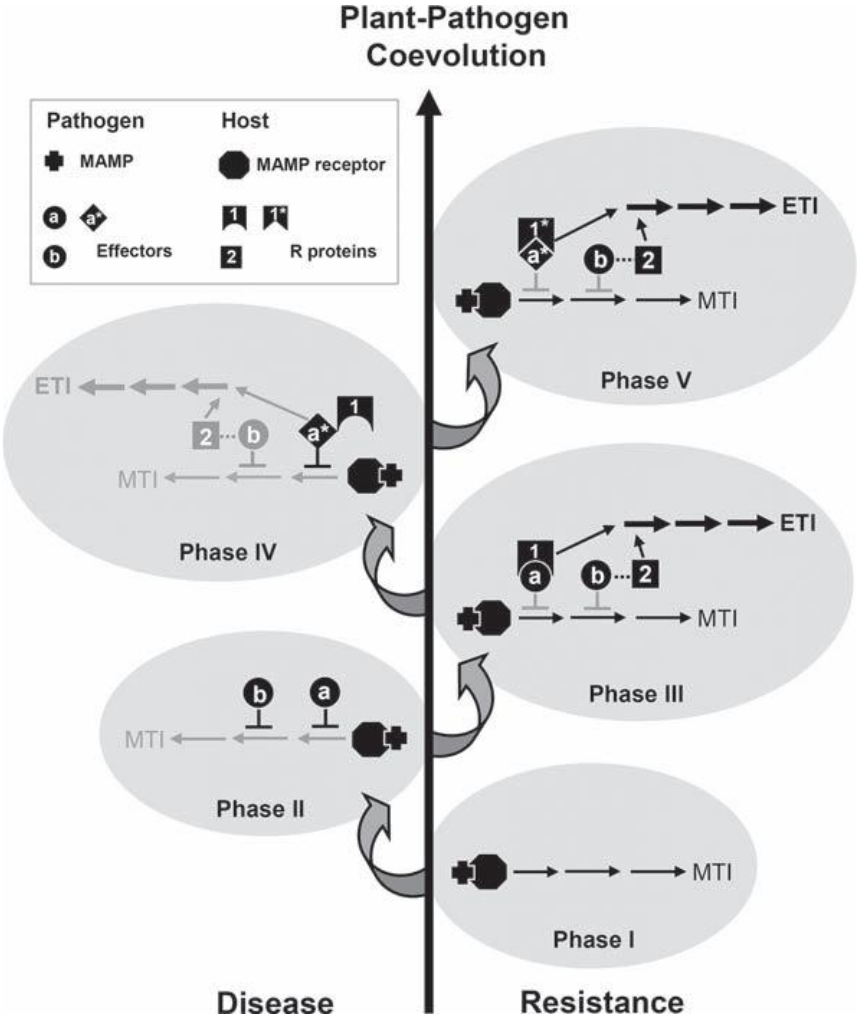

Figure I. 24 A general model for plant–pathogen coevolution with a multilayered plant defense system. Non-host resistance (*Phase I*); Pathogen effector proteins "*a*" and "*b*" can eventually overcame non-host resistance (*Phase II*); Evolution of specific R proteins "*1*" and "*2*" in plants to recognize the effector protein inducing a stronger defense response (*Phase III*). The pathogen accumulated mutations that change the structure of effector "*a*" to escape recognition by R protein "*1*", resulting in suppression of R-resistance. In the other scenario, effector gene "*b*" might have been deleted in the pathogen population if this incurred little fitness penalty in virulence, or "*b*" became rare in the pathogen population as a consequence of *R*-resistance, which in turn relaxed the selection for *R* gene "*2*", resulting in a low frequency of *R* gene "*2*" in the plant population as a result of balancing selection for the benefit and cost of *R* gene expression (*Phase IV*). Next, the plant faced pressure to evolve a new *R* gene "*1*"* to recognize "*a*"* to activate defense; or as the frequency of the effector gene "*b*" increased, the frequency of *R* gene "*2*" also increased due to its selective advantage in the presence of "*b*" (*Phase V*). From: Xiao et al. (2008).

However, according to the population genetics theory of selective sweeps, the rapid turnover of new R-gene specificity should cause a reduction in the age and number of alleles at a locus which is contradictory to the presence of ancient and many segregating alleles observed in R-gene plant population. This suggested that a microevolutionary mechanism for promoting the maintenance of stable polymorphism exist (Stahl et al., 1999). This observation led to the proposition of the second model of R gene evolution: the **trench warfare** model where R genes are maintained in the population by balancing selection over long periods (Stahl et al., 1999). Thus, frequencies of R genes in natural plant populations are balanced by frequency-dependent selection. An R gene becomes prevalent as a result of its selective advantage, whereas the frequency of such R gene is reduced when the corresponding pathogen causes less disease pressure (Van der Hoorn et al., 2002). Balancing selection results in a balanced polymorphism of R genes and can maintain Avr–R gene pairs over an indefinite period of time (Van der Hoorn et al., 2002).

The *RPM1* (Stahl et al., 1999) and *RPS2* (Mauricio et al., 2003) loci of *Arabidopsis* in which variation is low with no evidence of diversifying selection between functional and nonfunctional forms represents this model. Different from positive selection, balancing selection increases levels of polymorphism at linked sites by several mechanisms including over-dominance, frequency-dependent selection, and temporally or spatially variable selection (Tiffin and Moeller, 2006).

Although balancing selection can explain a number of ancient alleles in plant population, this model only cannot explain the generation of new R genes specificities. Therefore, R gene dynamics in a natural plant population probably reflect a combination of balancing selection and birth and death models, the latter perhaps being relatively slow (Van der Hoorn et al., 2002).

Convergent selection (i.e. the acquisition of the same biological trait in unrelated lineages) rather than balancing selection was proposed to explain how R genes from different plant species can recognize the same pathogen. This is the case of the *Rpg1*-b from *Glycine max* (soybean) and *RPM1* from *Arabidopsis thaliana* (Asfield et al., 2004). Both genes belong to CNL class and confer resistance to races of the same type III effector protein from *Pseudomonas syringae*, AvrB. However, the genes share only 34% identity across the nucleotide-binding domain and are even more divergent in the LRR domain. Phylogenetic analysis demonstrated that these genes belong to paralogous lineages that arose from gene duplications that occurred before the monocot–dicot split. Considering the degree of sequence divergence and evolutionary distance between *Rpg1*-b and *RPM1*, it is unlikely that their recognition specificities are shared by descent. In this case, recognition specificities of these two genes appear to have evolved independently as a result of the selection pressure imposed by P. syringae (Asfield et al., 2004; McDowell, 2004).

7.2 Evolutionary patterns R genes clusters

Two types of NBS-LRR gene evolution were distinguished from analysis of the sequence diversification rate at two complex R clusters, RGC2 in lettuce and R1 in *S. demissum*, (Kuang et al. 2004, 2005). Type I comprises fast-evolving genes, characterized by chimeric structures resulting from frequent sequence exchanges among group members and consequently lacking a clear allelic/orthologous relationship between different genotypes. The Type II comprises slow-evolving genes, exhibiting infrequent sequence exchanges between paralogous sequences and obvious allelic/orthologous relationships among different genotypes (Kuang et al. 2004, 2005). (Figure I.25).This heterogeneous rate of evolution is consistent with a birth-and-death model (Michelmore & Meyers, 1998; Kuang et al., 2004). Individual paralogs of type II of R genes (i.e. alleles or orthologs) are highly conserved among accessions. Some NBS–LRR-encoding genes in *A. thaliana* seem to have evolved in a pattern similar to that for type II R genes; these genes are not chimeras of different paralogs and are highly conserved (usually exhibiting >99% nucleotide identity) in different genotypes (Bakker et al., 2006).

Type I genes can be stochastically converted into Type II genes by structural rearrangements that inhibit mispairing and thereby reduce sequence exchange. At least a subset of Type II genes is conserved because they have evolved important resistance specificities (Kuang et al., 2005). By contrast, some Type I genes might be works-in-progress. Consistent with this idea, RGC2 Type I genes appear to be subjected to strong diversifying selection while Type II genes are under purifying selection (Kuang et al., 2004). However evolutionary analyses of NBS–LRR genes sometimes can be complicated by extensive gene duplication, variable rates of sequence exchanges between paralogs, and indistinguishable orthologous relationships (Cadwell and Michelmore, 2009). However evolutionary analyses of NBS–LRR genes sometimes can be complicated by extensive gene duplication, variable rates of sequence exchanges between paralogs, and indistinguishable orthologous relationships (Cadwell and Michelmore, 2009).

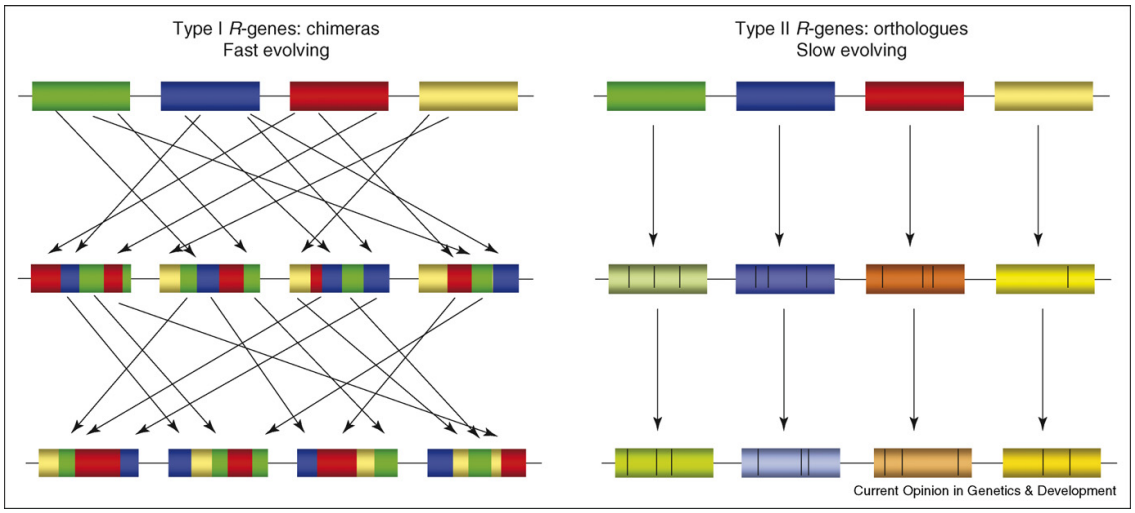

Figure I. 25 Evolutionary pattern of R genes. 'Fast evolving' type I resistance genes are characterized by frequent sequence exchanges between paralogs that obscure orthologous relationships, whereas 'slow evolving' type II resistance genes rarely experience sequence exchanges between paralogs and maintain orthologous relationships. Black arrows represent changes accrued over evolutionary time. Point mutations are shown as vertical white lines. From: Fredman and Baker, 2007.

8. Wide genome analysis of NBS-LRR encoding genes

The complete or partial plant genome sequencing revealed that genes encoding NB-LRR proteins represent one of the largest gene families in plants. The predicted number of NBS-encoding R identified in the genome of different plants is shown in Table I.10. As aforementioned, the deduced NB-LRR proteins can be divided into two subfamilies, TIR-NBS-LRR (TNL) and non-TNL based on their N-terminal features. Non-TNL proteins often have a putative N-terminal coil-coil (CC) and are thus designated CNLs (Dangl and Jones 2001).

NBS-LRR-encoding genes are found in non vascular plants and gymnosperms as well as in angiosperms (Akita & Valkonen, 2002; Zhou et al., 2004). In genome of dicot plants both subfamilies of NBS-LRR-encoding genes were found (Rickly et al., 2002; Meyers et al. 2003; Yang et al., 2008; Ameline-Torregrosa et al., 2008; Ming et al., 2008) while in monocot only non-TNL genes were identified (Bai et al. 2002; Meyers et al., 2002; Zhou et al., 2004, Monosi et al., 2004; Paterson et al., 2009). A few TN genes (TNL but lacking an LRR domain) have been identified in rice (*Oryza sativa*) but greatly differ from typical TNL genes (Bai et al., 2002; Zhou et al., 2004). It suggests that TNL genes might have evolved before separation of the dicot and monocot genomes and they were lost during monocot evolution (Meyers et al., 2002).

Table I. 10 The total number of predicted NBS-encoding genes identified in the sequenced angiosperm genomes.

Specie	Type of NBS-LRR			Total NBS-enconding gene	Genome size Mb	% NBS-LRR in genome	Reference
	TNL	NON-TIR (CC or X)	other				
A. thaliana Col 1	94	55		149	125	0.53	Meyeres et al., 2003
Oryza sativa Var. Nipponbare	-	480	3-TIR-NBS Lack of LRR	535		1	Zhou et al., 2004
Oryza sativa Var. japonica	-	272	50	508	390	1.35	Li et al., 2010
Oryza sativa		489		581		1	Monosi et al., 2004
Vitis vinifera	97	203	159	459	487	1.51	Yang et al.,2008
Populus trichocarpa	78	252		330	485	0.72	Yang et al., 2008
Brassica rapa[a]	42	17	33	92	~100[a] (529)		Mun et al., 2009
Sorghum bicolor	-	184	61	245	730	0.68	Li et al., 2010
Sorghum bicolor		209	2	211	~ 730		Paterson et al., 2009
Carica papaya	6	4	44	54	372	0.2	Porter et al., 2009
Lotus japonica[a]	174	55		229 (60% genome)	~315[a] (472)		Sato et al., 2008
Medicago trunculata[a]	156	177	30	333 (58% genome)	~186[a] (500)		Ameline-Torregrosa et al., 2008
Zea mays	-	95	34	129	2300	0.4	Li et al., 2010
Brachypodium distachyon	-	212		239	350	0.74	Li et al., 2010

[a] The NB-encoding genes of Br, Mt, Lj were identified based on partial genome sequences. Overall genome sizes are indicated in parentheses

Phylogenetic analyses indicated that separation of the TNL and CNL subfamilies represents an ancient division that may date back to the common ancestor of angiosperms and gymnosperms around 300 million years ago (Meyers et al., 1999; Young et al., 2000) and that members of the TIR subfamily are relatively more conserved, whereas members of the non-TIR subfamily are more divergent (Cannon et al. 2002; Meyers et al. 2003). The TNL genes were also be found in the moss *Physcomitrella patens* genome suggesting that the origin of NB-LRR sequences is before divergence between Bryophytes and Ptéridophytes (~400 Mya) (Palmer *et al.*, 2004; Rensing *et al.*, 2008; Cuming, 2009).

Evolutionary studies using 1600 NBS sequences from 23 plant families suggest that CNLs are likely to be the more ancient subclass of R genes (Figure I.26). First of all, because CNLs from monocots and dicots cluster together, indicating that angiosperm ancestors had a common origin (McHale et al., 2006). Secondly, the conserved intron positions of the TNLs and the shorter

phylogenetic branch lengths within the TNL phylogeny suggest a shorter evolutionary history (Cannon et al., 2002; Meyers et al., 2003).

Comparative analysis of NBS genes in four plant genomes (*Arabidopsis*, rice, grape, and poplar) revealed extensive species-specific expansion in TNLs genes (Yang et al. 2008). In contrast, non-TNLs did not segregate by species but mostly clustered in interspecific phylogenetic clades. These data suggested different evolutionary rates and patterns between TNLs and non-TNLs (Yang et al. 2008). Besides that, phylogenetic trees using the NBS domain confirmed the recent duplications of NBS genes in poplar and grapevine since short branches from the terminal nodes were more frequent in these two perennial species than those in *Arabidopsis* and rice (Yang et al., 2008).

In gramineous species a comparative analysis identified: 129, 245, 239 and 508 NBSs genes in maize, sorghum, brachypodium and rice, respectively, suggesting considerable variations of these genes. All gramineous NBS genes are supposed to have originated from common ancestors (Li et al., 2010). Although NBS–LRR genes derive from a common ancestor, the numbers of NBS genes are quite different among the four grass species (maize, sorghum, brachypodium and rice). The maize genome (2,300 Mb; Schnable et al. 2009) is much larger than sorghum (730 Mb; Paterson et al. 2009), brachypodium (350 Mb; Huo et al. 2008) and rice 390 Mb (International Rice Genome Sequence Project 2005), indicating that the total number of NBS–LRR genes is not associated with genome expansion in the grass species. In this scenario, the variation in the number of NBS-LRR genes among gramineous species must be caused by gene loss or expansion within a species (Li et al., 2010).

The NBS-LRR genes range from 0.2 to 1.51 % of all predicted genes in papaya and grapevine, respectively (see Table I.10). The small number of NB-LRR genes in papaya genome could be attributed to lack of recent genome duplication in this species (Ming et al. 2008). For example in papaya the number of predicted genes is 24,746 and only 0.2% encodes NBS domains while in *A. thaliana* from 25,500 predicted genes 0.53% represents NBS-encoding gene (Meyeres et al., 2003). Thus, a significant proportion of the plant genome appears dedicated to NBS-LRR-based pathogen surveillance, and NBS-LRR gene duplication has had a tremendous impact on the evolution of plant immunity (McDowell and Simon et al., 2006).

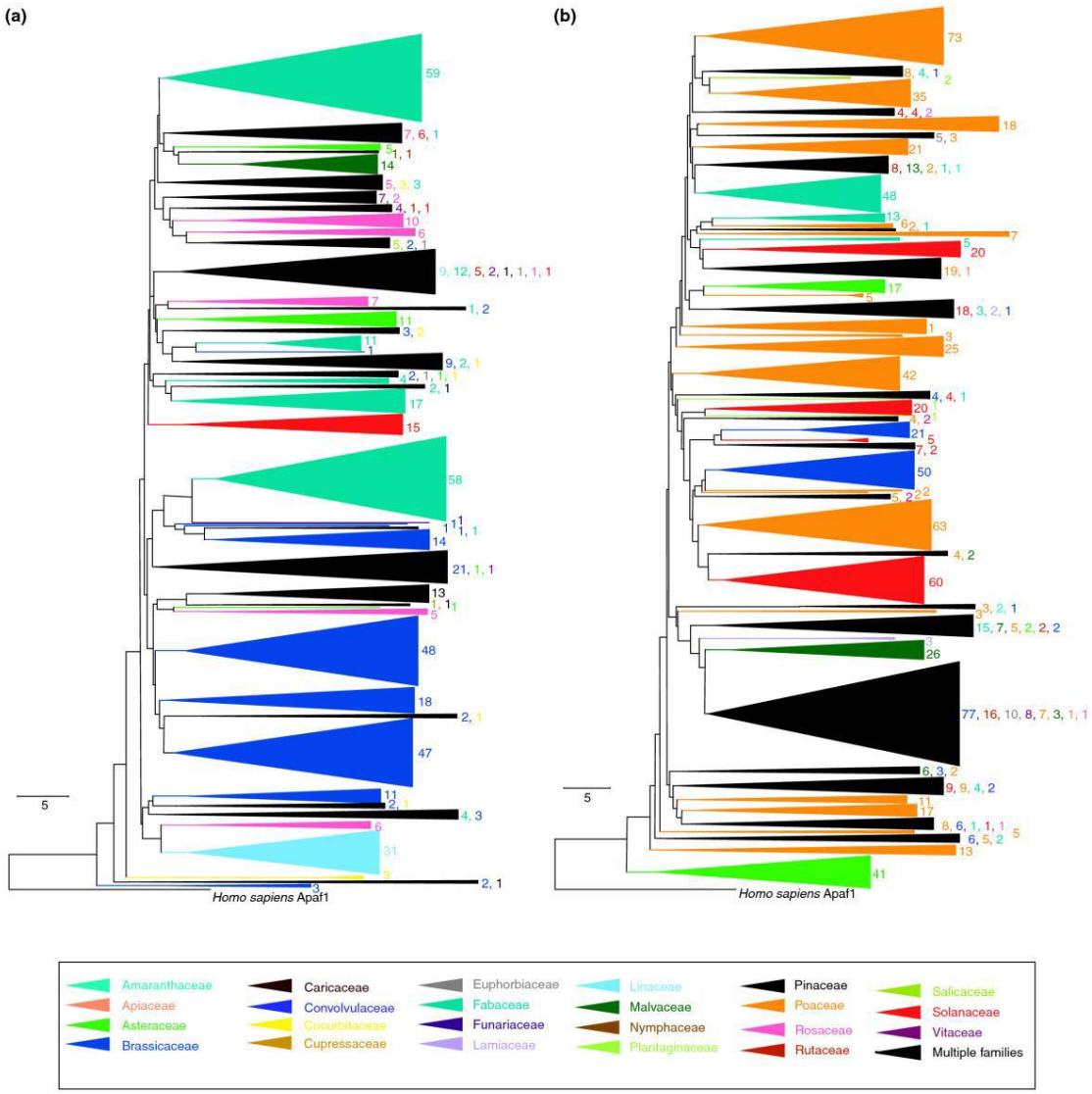

Figure I. 26 Neighbor-joining tree showing the family-specific amplification of NBS sequences. (a) TNLs. (b) CNLs. The complete tree was based on 1,600 sequences Clades that contained sequences from individual plant families were collapsed into single branches and the number of sequences in each branch is indicated. Different taxa are assigned different colors; clades with representatives from several families are shown in black. The scale bar represents five nucleotide substitutions. From: (McHale et al., 2006).

8.1 Organization of NBS-LRR genes into plant genomes

The NBS-LRR genes are organized in single-gene loci, clusters, and superclusters (Meyers et al. 1999; Pan et al., 2000; Wendel and Fluhr 2000; Rickly et al 2002; Zhou et al. 2004; Ameline-Torregrosa et al. 2008; Yang et al., 2008).

A gene cluster was defined by Holub (2001) as a region in which two neighboring homologous genes are <200 kb apart. R clusters range in size from two tandem paralogs to large complexes spanning several megabases (Fredman & Bakers, 2007). The largest R clusters characterized to date include the maize Rp1 cluster up to 52 homologs per haplotype (Smith et al., 2004). The clusters can be simple (homologous R-gene sequences arising from a single gene family) or complex (derived from two or more unrelated families) and may also contain unrelated single genes interspersed between the homologs (Fredman & Bakers 2007). Usually, only a single gene in the cluster determines resistance as shown by mutant or transgenic complementation analysis. The genetic and hybridization data indicate that resistance genotypes should be considered as haplotypes rather than individual genes (Michelmore & Meyers, 1998).

The 149 Arabidopsis NBS-LRR genes are distributed in 40 clusters ranging in size from two to eight genes, while 40 genes exist as singletons (Richly et al., 2002; Meyers et al., 2003) (Figure I.27). More than a than half of these single-copy genes are predicted to be either duplicated in or absent from the genomes of other accessions of *A. thaliana* (Bakker et al., 2006). The pattern of R gene organization in *A. thaliana*, suggested that after an ancient event which generated CC- and TIR-type classes, a few ancestral genes underwent local amplification, leading to tandem gene pair, which could have been broken up by chromosomal translocation or by other types of gene selection (Rickly et al, 2002).

The rice genome contains an approximately equivalent proportion of clustered genes and non clustered genes (Bai et al., 2002; Monosi et al., 2004; Zhou et al., 2004). In the *M. truculata* genome 79.8% of NBS domains occur in clusters of at least two genes and 49.5% in clusters of at least five genes. The same pattern of distribution has been observed in the grapevine (83.2%) and the poplar (67.5%) genomes (Yang et al., 2008).

The physical clustering suggests that tandem duplication is an important source of new R genes, and this hypothesis is supported by two trends. First, genes within a cluster tend to occupy the same phylogenetic lineage. Second, gene copy number can vary widely among haplotypes within a species (McDowell & Simon, 2006). An example is the maize *Rp1* rust resistance locus, in which the number of *Rp1* paralogues in different haplotypes varied from a single gene to >50 genes (Smith et al., 2004). Such variation in gene copy number has probably arisen due to mispairing and unequal crossing over between clustered NB-LRR genes, or between repetitive sequences such as retrotransposons that are frequently present in intergenic regions of *R* gene clusters (Hammond-Kosak & Kanuya, 2007).

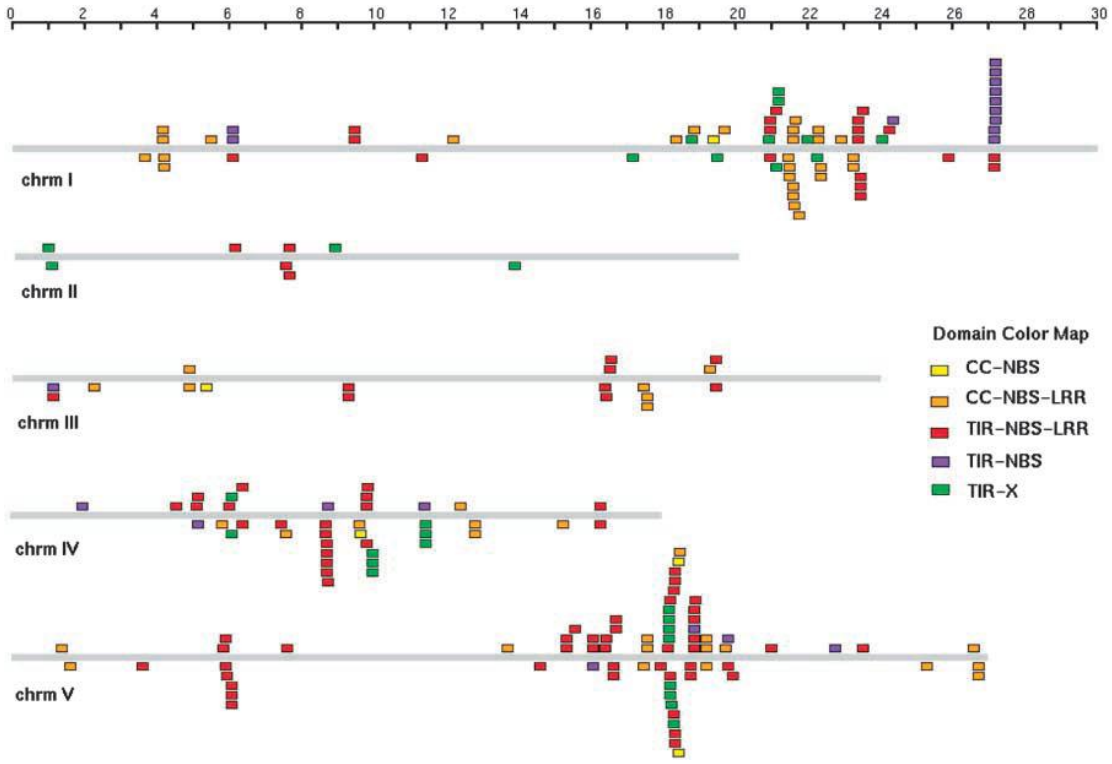

Figure I. 27 Physical locations of Arabidopsis sequences that encode NBS proteins similar to Plant *R* Genes. Boxes above and below each Arabidopsis chromosome (chrm; gray bars) designate the approximate locations of each gene. Chromosome lengths are shown in megabase pairs on the scale at top (Meyers et al., 2003).

9. Mechanisms of R-genes diversification

Several mechanisms are involved in diversification of NBS-LRR genes. According to Leister, (2004) they can be grouped into three main classes: One involves ***Duplication*** such as tandem, segmental and ectopic duplication. Other is result of ***Recombination*** such as unequal, equal crossing over or yet inter, intragenic (gene conversion) and concerted evolution. And another derived from positive, negative or diversifying ***Selection*** (Figure I.28). Each of these mechanisms will be detailed below.

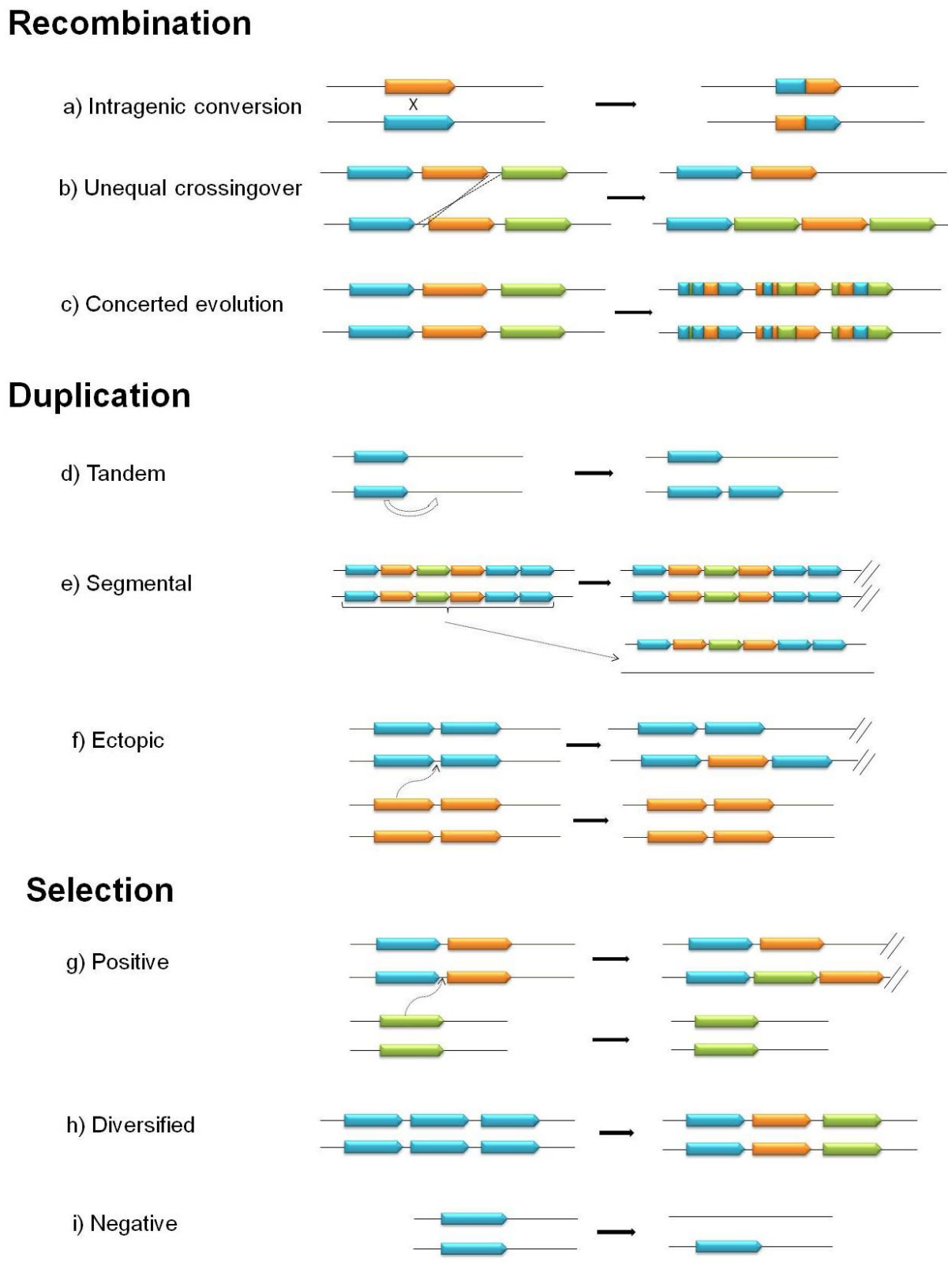

Figure I. 28 The mechanisms that contribute to diversity of *R* genes and their loci. Adapted from: Leister et al. 2004.

9.1 Duplication

Duplication is considered as the birth of an R gene (Huang et al., 2009). It can create new loci, alter gene family number through recombination, or generate repeated sequences within a gene (Richter & Ronald, 2000). Duplication of R genes can occur by ***tandem duplication*** where the duplicated segment is transferred to a site contiguous to the original one, by ***ectopic duplication*** that in contrary to tandem duplication, the individual or small groups of genes are moved to an unlinked locus, or by ***segmental duplication*** where large stretches of DNA containing many genes are duplicated to an unlinked locus but in a large-scale when compared to ectopic duplication (Leister, 2004).

Recent local expansion by tandem duplication within a local chromosomal region has demonstrated to play a major role in NBS gene expansion in *Arabidopsis* (Rickly et al., 2002; Baumgarten et al., 2003), *M. trunculata* (Ameline-Torregrosa et al, 2008), *B. rapa* (Mun et al., 2009), grapevine and poplar (Yang et al., 2008). Around 80% of duplications events in *A. thaliana* occur within relatively restricted chromosomal regions while the remaining 20% involve duplication to new chromosomal locations (Baumgarten et al., 2003).

Although local tandem duplication explains the origin of a large fraction of NBS-LRRs, genome surveys have revealed 'mixed clusters' comprising evolutionarily distant NBS-LRR genes. For example, at least ten clusters in *Arabidopsis* contain interspersed CNLs and TNLs (Richly et al., 2002; Meyers et al., 2003). Two models have been proposed to explain NBS-LRR distribution patterns that are inconsistent with simple tandem duplication. The 'rapid rearrangement' model emphasizes the importance of ectopic duplications, in which single genes or small groups are transposed to distal locations by an undefined mechanism (McDowell and Smith, 2006). Candidate mechanisms for ectopic duplication are recombination events between homologous sequences at unlinked sites, or the effects of the action of transposable elements (Leister, 2004). This model is supported by an apparent lack of large-scale synteny in sequences flanking related NB-LRR genes in cereals and Arabidopsis (Leister, 2004). A different but non-exclusive, 'conserved synteny' model emphasizes the importance of large-scale segmental duplication with subsequent local rearrangement (Leister, 2004).

9.1.1 Duplication by polyploidization

Polyploidy is the first mechanisms by which genes are duplicated. Several studies have revealed that multiple rounds of polyploidy have occurred during angiosperm evolution (Adams & Wendel, 2005). The polyploidization phenomena involve a whole spectrum of molecular and

physiological adjustments. Extensive genomic rearrangements, including exchanges between genomes and gene loss, often arise with the onset of polyploidization (Levy & Feldman, 2004). Recombination between homoelogous chromosomes might create lineages that are reciprocally distinguished not just for single genes but for whole chromosome arms or chromosomes segments (Osbom et al., 2003). Genes that are duplicated by polyploidy could be expressed at equal levels, or there could be unequal expression or silencing of one copy (Adams & Wendel, 2005). A duplicated gene may be lost, inactivated, or develop a new function (Kellis et al. 2004) (Figure I. 29).

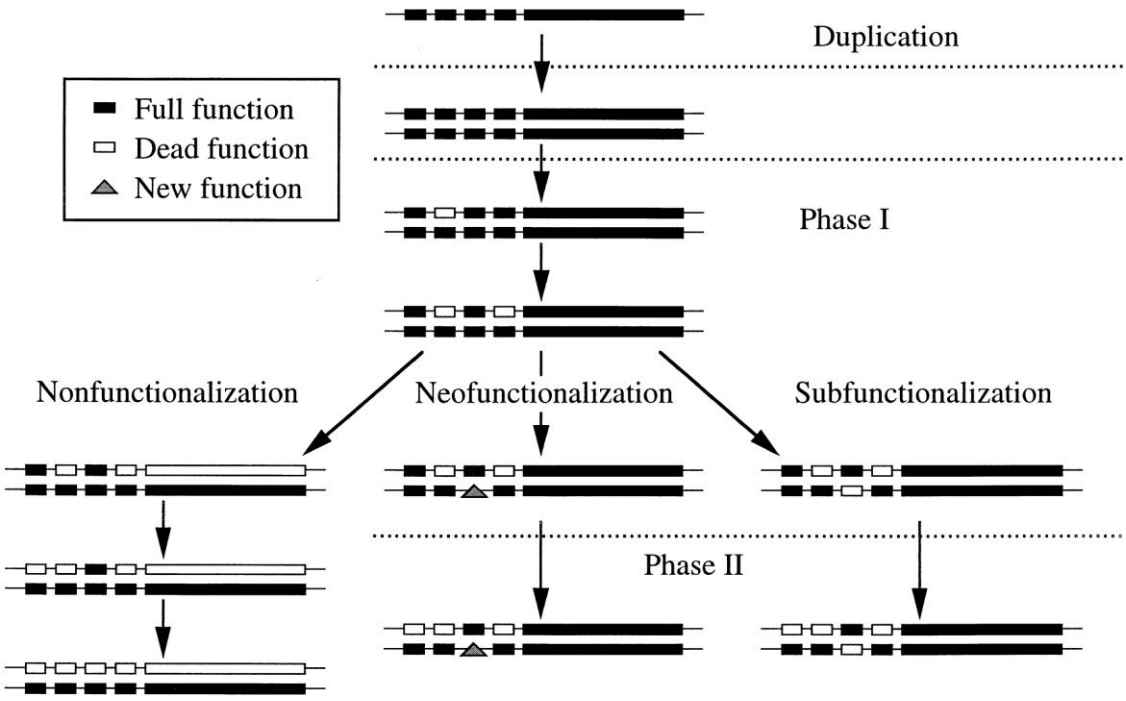

Figure I. 29 Three potential fates of duplicate gene pairs with multiple regulatory regions. In the first two steps, one of the copies acquires null mutations in each of two regulatory regions. On the left, the next fixed mutation results in a nonfunctional pseudogene, the remaining regulatory regions associated with this copy eventually accumulate degenerative mutations. On the right, the lower copy acquires a null mutation in a regulatory region that is intact in the upper copy. Because both copies are now essential for complete gene expression, this third mutational event permanently preserves both members of the gene pair from future nonfunctionalization. The fourth regulatory region, however, may still eventually acquire a null mutation in one copy or the other. In the center, a regulatory region acquires a new function that preserves that copy. If the beneficial mutation occurs at the expense of an otherwise essential function, then the duplicate copy is preserved because it retains the original function. From: Force et al., 1999.

9.2 Sequence recombination

There are two types of ***unequal crossing over***: Intergenic recombination between distinct genes and intragenic recombination (or ***gene conversion***) that occurs among different sites within the same gene locus. Intergenic unequal crossover (has the potential to alter gene expression, whereas intragenic mispairing generates chimeric genes that may encode novel functions (Fredman & Bakers 2007). Both types of unequal recombination will also result in altered gene copy number within the cluster according to the number of genes present in the region between the mispaired recombination sites (Fredman & Bakers 2007). Unequal crossing-over following meiotic mispairing and, to a lesser extent, gene conversion are the major mechanisms involved in generating novel resistance specificities (Richter et al. 1995; Hammond-Kosack and Jones 1997; Hulbert 1997; Parniske et al. 1997).

The frequency of intergenic sequence exchanges detected between NBS-LRR in *A. thaliana* is much higher when they were located in the same 2-Mb chromosomal region than when located in different chromosomal regions. Sequence exchanges were also evident among similar NBS-LRR genes located in different genomic regions, but they were less frequent (Baumgarten et al. 2003). The frequency of gene conversions between two homologs is usually negatively correlated with distance and positively correlated with sequence similarity (Shen and Huang, 1986; Semple and Wolfe, 1999; Mondragon-Palomino and Gaut, 2005).

Sequence exchanges have been documented in several R-clusters (Parniske & Jones, 1999; Kuang et al., 2004; Mondragon-Palomino & Gaut, 2005) and are associated with genic diversity, characterized by sequence shuffling and chimeric genes, and haplotypic diversity, characterized by a variable number of R-homologs within the cluster and a general loss of syntenic/orthologous relationships between haplotypes (Cadwell and Michelmore, 2009).

In gene conversion, one gene is the donor and gives part or all of its information to another gene, the acceptor. The process is done through strand breaks and sequence exchanges on the biological level and typically occurs between highly similar genes, for instance, genes that belong to the same gene family (Lawson et al., 2009). Naturally, this leads to an alteration in the acceptor gene (the donor remains unchanged). If only a part of the acceptor sequence is replaced it could lead to a change in functionality in the acceptor gene and it represents an important method to achieve genetic diversity (Lawson et al., 2009). In this case pseudogenes could also act as donor contributing to diversity. In many cases, only a specific region within the gene (for instance the second exon) is involved in the conversion (Lawson et al., 2009). Due to the repeated structure of the LRR domain, this region of the gene is either the most susceptible or the most permissive region for unequal crossing over (Meyers et al., 2003).

However, frequently gene conversion followed by recombination can lead sequence homogenization, and thus lead to ***concerted evolution*** of *R* genes paralogs within clusters (Michelmore and Meyers 1998; Parniske and Jones 1999; Young, 2000). Concerted evolution can be defined as the process by which a series of nucleotide sequences or different members of a gene family remain similar or identical through time (Leister, 2004). In that case paralogs copies (i.e gene duplicated within a genome) are more similar to one another than to their orthologs (i.e genes in different species that evolved from a common ancestral gene by speciation) in other subspecies (Wang et al., 2007). Extensive concerted evolution has been found in two *indica* and *japonica* subspecies of rice as a result of both conversion and crossing over, among genes duplicated by segmental/genome wide events, and proximally duplicated genes (Wang et al., 2007).

Frequent sequence exchanges among homologs have homogenized intron sequences of some R-gene families, resulting in a higher level of conservation in introns than their flanking exons (Kuang et al., 2004, 2005). However, diversifying selection has been predicted to maintain the variation introduced through sequence exchange, which may explain the lack of concerted evolution (Kuang et al., 2004). It has been suggested that gene conversion be a possible mechanism to explain sequence and functional conservation among paleologs (ancient, duplicated genes at homeologous locations) in the *Arabidopsis* and rice genomes after large-scale duplication events (Chapman et al., 2006).

Interestingly, gene conversion between NBS-encoding paralogs in perennial species (grapevine and poplar) appeared to be more frequent than in annual plants (*Arabidopsis* and rice) (Yang et al., 2008). The whole comparison of NBS-encoding in those species revealed that 3–10-fold more gene conversion events was detected in perennial than in annual plants suggesting that divergent evolutionary patterns between the annual and perennial plants can exist (Yang et al., 2008). Compared with annual plants, the long-generation time of woody species prevents a comparable rate of evolutionary change relative to microbial or insect pests. However, the authors suggested that the higher homologous recombination rate together with a significant excess of recent duplications found in grapevine and poplar could compensate for life history traits and generate novel resistance profiles (Yang et al., 2008).

9.3 Selection

The ‘neutral theory’ states that the observed genetic variation within species, as well as divergence between species, is caused by the random fixation of neutral mutations (King and Jukes, 1969; Kimura, 1983). Detecting adaptive evolution involves comparing amino acid substitution rates (*K*a) to synonymous substitution rates (*K*s) in the same gene. Under the assumption that synonymous changes approximate the neutral rate of molecular evolution, ***neutral*** mutation is assigned when Ka

equals to Ks, ***negative selection*** (or purifying) when Ka is less than Ks, and ***positive selection*** (or diversifying) when Ka exceeds Ks. Therefore, statistics of the two variables in genes from different evolutionary lineages provides a powerful tool for quantifying molecular evolution (Bergelson et al., 2001; Zhang et al., 2006).

Because of the coevolutionary arms race between hosts and their pathogens, genes involved in their interaction are expected to evolve under ***positive selection***. Strong positive selection (the process by which new advantageous genetic variants sweep a population) and frequent recombination were found to be the major forces for a rapid evolution and diversification of *R*-genes (Chen et al. 2010).

Strong positive selection has been detected for at least two low copy genes, *RPP13* in *Arabidopsis thaliana* and *L* in flax, in which sequence exchange between paralogs has been rare (Ellis et al. 1999; Rose et al., 2004; Dodds et al., 2006). Furthermore, several single-copy NBS–LRR encoding loci have presence–absence polymorphisms that correlate with resistance/susceptibility phenotypes, limited diversity within each allelic class, and ancient polymorphisms (Stahl et al., 1999; Tian et al., 2002; Shen et al., 2006). Frequency-dependent selection is thought to generate and maintain the diversity at R-gene loci exhibiting both patterns of variation (Michelmore and Meyers 1998; Stahl et al. 1999).

A strong preference for nonsynonymous compared to synonymous codon substitutions has been detected in many plant R gene families (Ellis et al. 2000; Jones and Dangl 2006; Bent and Mackey 2007). Functional analysis of R genes has proved that nonsynonymous replacements in the xxLxLxx motif are sufficient to disrupt or alterate gene function. The functional importance of LRRs as the domains of specificity is further supported by results from domain swaps and mutational analyses of R genes (Warren et al., 1998; Ellis et al., 1999; Hwang et al., 2000; Luck et al., 2000; Dodds et al. 2001; Dodds et al., 2006; Geffroy et al., 2009). As previously explained, R gene specificity appears to reside in the solvent-exposed residues in the xxLxLxx motif that is located within the LRR region. Additionally, evidence of positive selection is found in pathogen molecules involved in host pathogen co evolution (Allen et al. 2004; Rose et al., 2004; Dodds et al., 2006).

A wide analysis of *Arabidopsis* genomes demonstrates that at least 17.4% out of the total 149 R genes are under positive selection (Meyers et al. 2003) while in rice only 11.2% of R genes analyzed between two rice cultivars are likely under positive selection (Yang et al., 2008). This indicates a lower proportion of R genes in rice under positive selection compared to *Arabidopsis* (Yang et al., 2008).

The Ka/Ks ratio was significantly higher in the non-TIR NBS than the TIR NBS families, in grapevine and poplar suggesting stronger diversifying selection in the non-TIR-NBS subfamily (Yang et al., 2008).

9.4 Other mechanisms that contributed to generate R gene diversification

a) Illegitimate recombination

Illegitimate recombination (IR) is a process characterized by the recombination between non-homologous or short homologous sequences (van Rijk & Hans Bloemendal, 2003). IR can occur between dispersed homologous sequences of only a few (usually 1–10) base pairs (Devos et al., 2002; Daley et al., 2005). This asymmetric pairing followed by sequence exchange can result in either duplications or deletions (van Rijk & Hans Bloemendal, 2003). IR has been proposed as a new major evolutionary mechanism at the basis of variability of LRR domains (Wicker et al., 2007). The complex repeat arrays in four RGA lineages (*Cf-2, RGH2, Pm3* and *Xa1*) were demonstrated to be a result of initial simple duplications caused by IR (Wicker et al., 2007). IR can also generates InDels and point mutations in R genes as was indicated by the analysis of conserved small repeats flanking the InDels in *Pm3* alleles from wheat that confers resistance to powdery mildew caused by *Blumeria graminis* sp. tritici) (Bhullar et al., 2009).

b) Insertion/deletion

LRR domains are amenable to expansion and contraction by insertion or deletion of complete LRR units. Deletion or insertion of complete LRRs may occur by intragenic crossing over. This was revealed in a mutant allele of *RPP5* that has an in-frame intragenic duplication of four LRRs without loss of function (Parker et al., 1997).

In other example the locus *RPP1* from *Arabidopsis* that has three alleles and the only difference among the alleles is a deletion of a complete LRR in one *RPP1* member. This member demonstrated to be resistant to only one of the three races of *Peronospora parasitica*. The deletion of a unit of LRR domain potentially gives rise to novel recognition surfaces (Botella et al., 1998).

Deflection or insertion in LRR domain are associated with altered specificity in the *L* and *M* loci in flax (Anderson *et al.* 1997; Ellis *et al.* 1999; Luck *et al.* 2000), in the *Cf-5* locus in tomato (Dixon *et al.* 1998), and in the *RPP5* locus in Arabidopsis (Noël *et al.* 1999). A clear example could be demonstrated with *Cf* genes from tomato that confer resistance to strains of the fungal pathogen *Cladosporium fulvum. Cf-4* and *Cf-9* recognize specific races carrying AVR4 and AVR9 respectively. Experiments of insertion and deletion in LRR domain revealed that variation in LRR number is directly involved in gene specificity (Van der Hoorn *et al.*, 2001; Wulff *et al.*, 2001). A *Cf-9* mutant

that had two unit of LRR deleted (11th and 12th) lost the capability to recognize AVR9, but acquired the capability of recognize AVR4 (Van der Hoorn *et al.*, 2001).

c) **Transposons**

Transposable elements of several types are major components of most plant genomes and are also present in clusters of resistance genes. Such elements could play several roles in the evolution of resistance genes. Insertion of the same element into two positions flanking a *R* gene could provide the primary duplicated sequences allowing unequal crossing-over and the initial duplication of the *R* gene sequence (Wessler et al., 1995). Insertions will tend to increase misalignment and therefore increase the chances of unequal crossing-over when hemizygous. However, when homozygous, insertions will tend to decrease the chances of misalignment and therefore contribute to the divergence of intergenic regions (Michelmore and Meyers, 1998).

Insertion of mobile elements also contributed to generate repeats that offer new possibilities of mispairing during recombination, giving rise to unequal crossovers and interlocus gene conversions (Richter & Ronald, 2000; Meyeres et al., 2003; McDowell & Simon, 2006). Extensive retropositions and chimerical genes were identified in rice genome, supporting that retroposition may be an important force in driving evolution of genes in grass species (Wang et al., 2006). In addition, 85.3, 31.6, 5.5 and 7.1% of transposable elements were detected in the 1-kb flanking regions of pseudo-*R*-genes in maize, rice, brachypodium and sorghum genomes, respectively (Li et al., 2010). Expansion of a TNL cluster by tandem duplications and insertions of retrotransposons has also been described for the *RPP4*/*RPP5* family (Noel et al., 1999).

Transposon-mediated transcriptional activation may play an important role in the refunctionalization of additional 'sleeping' R genes in the plant genome (Hayashi and Yoshida, 2009). The comparison of non-functional and a functional allele of *Pit* gene from rice that confers race-specific resistance against the fungal pathogen *Magnaporthe grisea* revealed that functional allele contains only four amino acid substitution and has the LTR retrotransposon Renovator inserted upstream. Functional analysis using chimeric constructs from both alleles demonstrated that the up regulated promoter activity conferred by the Renovator sequence is essential for Pit function (Hayashi and Yoshida, 2009).

d) **Pseudogenes**

Pseudogenes tend to evolve much faster than functional genes (Ota and Nei 1994) and therefore represent the possibility of more rapid evolution of new specificities (Michelmore and Meyers, 1998). Pseudogenes represent 10 and 3.5% of all predicated NBS-encoding genes in *A.*

thaliana (Meyers et al., 2003) and rice (Zhou et al., 2004), respectively, and they also provide a reservoir of genetic diversity that could be accessed by recombination or gene conversion (Meyers et al., 2003).

The recombination of two non-functional peseudogenes can create a new functional gene. This has been demonstrated by Huang et al. (2009) when they studied the origin of the wheat leaf-rust resistance gene *Lr21*. The *Lr21* is a single locus dominant gene that was introgressed from *Aegilops tauschii* into modern wheat by breeding. Analyzing sequences of *Lr21* and *lr21* alleles from 24 wheat cultivars and 25 accessions of *A. tauschii* three basic nonfunctional lr21 haplotypes, H1, H2, and H3, were identified. They were able to reconstitute a functional *Lr21* allele by crossing plants carrying the *H2* and *H1* alleles. A single resistant plant was found and proved to carry an intragenic recombination between *H1* and *H2*. From this finding the authors proposed a model called ‘‘death–recycle’’ where plants can reuse non functional alleles to create new resistance specificity for gene that are present at simple loci.

10. Patterns of R gene function

a) Single gene with multiple and functionally distinct alleles

Some R loci consist of only a single gene and can have multiple and functionally distinct alleles that recognize different races of a pathogen, for examples *L*, *P* and *M* locus from flax that recognize 13, 6 and 7 specificities from *Melampsora lini*, respectively (Ellis et al., 1997; 1999; 2007). Other example is the *Rpp13* locus of *Arabidopsis* that has 47 alleles for recognizing ATR13 effectors from *Hyaloperonospora arabidopsidis* (Hall et al., 2009).

b) Single copy genes conferring resistance/susceptibility by presence/absence

The RPM1 (Grant et al., 1995; Stahl et al., 1999) and RPS5 (Warren et al., 1995; Tian et al., 2002), RPS2, (Caicedo et al., 1999) genes from *Arabidopsis* controlling resistance to different strains of *Pseudomonas syringae* are examples of this pattern. In this case, there is no allelic variation in the loci; resistant genotypes contain a functional copy whilst in susceptible genotypes the gene is deleted. It has been proposed that these single copy R loci have evolved a very long time ago and now are subject to purifying selection, and therefore a reduction in gene diversity.

c) R genes from distant related species confer same specificity

R genes with the same specificity can be found in distant related species of plant as result of convergent evolution. For example: *Rpg1*-b from *Glycine max* (soybean) and *RPM1 Arabidopsis thaliana* (Asfield et al., 2004), both genes are from CNL class and confer resistance to races of the same type III effector protein from *Pseudomonas syringae,* AvrB.

d) R genes within a cluster can determine resistance to very different pathogens

R gene in a cluster that confer resistance to different pathogens are called **complex locus**.The locus *Rpg1* from soybean has several genes mapped in a 3cM interval that confer resistance against a diverse selection of pathogens including *Rpg1-b* and *Rpg1-r* effective against *Pseudomonas syringae, Rps3* effective against the oomycete *Phytophtora soja, Rsv1* effective against soybean mosaic virus and the *Rpv1* effective against peanut mottle virus (see Asfield et al., 2004). Other examples are available: two highly similar genes located a 115 kb apart on the potato chromosome 12 confer resistance to two unrelated pathogens, potato cyst nematode and potato virus X (van der Vossen et al., 2000). *RPP8* and *HRT* from *Arabidopsis* are paralogous genes that encode resistance to *Peronospora parasitica* and turnip crinkle virus, respectively (Cooleya et al., 2000). Similarly, the *Mi* gene from tomato encodes resistance to a nematode and an aphid species (Rossi et al., 1998; Vos, et al., 1998).

e) A cluster of R genes may contains sequences related in function but not in sequence

An example of this type of cluster is the Pto-Prf complex locus. The gene *Prf,* an NB–LRR gene, is within a cluster of five *Pto* homologs that encode protein kinases and both genes are required for resistance to *Pseudomonas syringae* pv. *tomato. Pto* and *Prf* act closely to regulate not only recognition of pathogen elicitor molecules, but also subsequent defense signaling, and their coordination function depends on their interaction in the plant cell (Salmeron et al., 1996; Pedley and Martin, 2003).

f) Two genes in a cluster can both encode the same specificity

For example, the *Cf2* gene from tomato has two functional sequences that encode the same specificity against the fungal pathogen *Cladosporium fulvum* (Dixon et al. 1996). The rice genes *Xa21* and *Xa21D*, which occur in the same complex locus provide resistance (partial in the case of *Xa21D*) to the same strains of the bacterial blight pathogen (Wang et al., 1998).

11. Comparative analysis of R genes

For single copy R genes the evolution rates can be determined from orthologous comparisons between species while in clustered R-genes it can be made by comparison among *R*-gene paralogs. Paralogs are created by a horizontal event (duplication) whereas orthologs reflect a vertical event (speciation) (Fitch, 1970).

a) Comparison of single copy genes

The estimates of the evolution rate have been obtained for three single-loci genes, *Rpm1*, *Rps2*, and *Rps5*, by comparing *Arabidopsis thaliana* and its congener, *A. lyrata*. For each of these *R*-loci, amino acid replacement changes have accumulated considerably more slowly than synonymous changes. Lower *K*a:*K*s ratios found in *Rpm1*, *Rps2*, and *Rps5* are compatible with adaptive evolution, however at a slower rate than that seen for complex loci (Chen et al., 2010).

To explore diversity at RPS2 locus from *A. thaliana* that governs resistance to strains of, *Pseudomonas syringae* pv. *tomato* expressing the *avrRpt2* gene, 17 accessions of *A. thaliana* were sequenced (Caicedo et al., 1999). Mutations occur throughout *RPS2* but are more frequent in the second half of the gene, in the region encoding the LRR. The *RPS2* gene exhibits one of the high levels of intraspecific sequence polymorphism where 1.26% of nucleotides are polymorphic within *RPS2*. Moreover, nearly half of the observed polymorphisms result in a change in amino acid composition and, of these, 70% are non-conservative changes. The resistant alleles are separated by only two to four nucleotide changes, and most of these changes result in amino acid substitution. The lack of resistance in all three susceptible classes of accessions can be explained by a defective *RPS2* allele (Caicedo et al., 1999).

The comparison of 13 alleles of *L* locus from flax revealed that some alleles have undergone large duplication or deletions in LRR region and that the alleles have a mosaic nature, probably due to variation present in ancestral alleles by recombination (Ellis et al., 1999).

b) Comparative analysis of R gene clusters across haploids or species

Important questions in genome evolution, particularly about the evolution of gene families and genome structure, can be addressed most effectively by analysis of large contiguous blocks of DNA

sequence from multiple species (Moreno et al., 2008). Comparative analyses of R-gene clusters across different haplotypes or species have demonstrated to be a worth approach to study gene family evolution. Several mechanisms of evolution were evidenced by such comparisons. Some examples are listed above:

The locus carrying out the *RPP5* gene was compared in different *Arabidopsis* ecotypes showing dynamic pattern of evolution including extensive recombination, retroelements insertion, point mutation, gene conversion and unequal recombination (Noël et al., 1999). The *R1* cluster was compared in three genomes of allohexaploid wild potato and demonstrated a fast evolve pattern by sequence exchange and retrotransposon insertion (Kuang et al., 2005). The comparison of three *Cf* genes clusters in different tomato species point to inter and intra locus recombination between *Cf* members (Parniske and Jones 1999; Kruijt et al., 2004).

To investigate whether *RPW8* locus has originated recently a comparison among the syntenic locus in *Arabidopsis thaliana*, *A. lyrata*, *Brassica rapa* and *B. oleracea* was performed (Xiao et al. 2004). The analysis showed that *RPW8* evolved by duplication and diversified selection in *Arabidopsis* after its separation from *Brassica* (Xiao et al., 2004).

The *Pm3* complex loci was compared from orthologous loci in the A genome of wheat at three different ploidy levels and in rice (Wicker et al. 2007). It was found an extremely dynamic evolution, resulting in minimal sequence conservation in *Pm3* loci from wheat and that it has evolved more rapidly than its homolog in rice.

The *RPP8* cluster was investigated in *A. thaliana, A. lyrata and A. arenosa* (Kuang et al., 2008). Both rapidly and slowly evolving *RPP8* homologs were identified. The sequence exchanges between fast evolving genes homologs within a locus was more frequent than sequence exchanges between homologs from two different loci, indicating the influence of chromosomal position on the evolution of these R genes. Moreover this study showed that differentiation between rapidly and slowly *RPP8* homologs occurred before the speciation of *A. thaliana, A. lyrata* and *A. arenosa* (Kuang et al., 2008).

The *Mi-1* cluster has been compared in nematode-susceptible tomato and in the *S. peruvianum* introgression in resistant tomato lines (Seah et al., 2007). Comparison of homologues suggests that extensive sequence exchange has occurred. Regions of diversifying selection were present in the ARC2 domain of the NBS region and dispersed throughout the LRR region, suggesting that these regions are possible locations of specificity determinants. Analysis of sequences flanking the *Mi-1*-homologues reveals blocks of homology, but complex differences were found in arrangement of these blocks when susceptible and resistant genotypes were compared indicating that the region has undergone considerable rearrangement during evolution, perhaps contributing to evolution of specificity (Seah et al., 2007).

The comparison of *Rpg*-1 locus from soybean and homologous region into three legumes (*G. tomentella, M. trunculata, P. vulgaris*) demonstrated that cluster expansion in soybean occurred by retroelement insertion (Innes et al., 2008).

The organization and evolution of *Pi2/9* locus (~100 kb) have been described in 4 wild species of rice representing three genomes AA, BB and CC: (*O. nivara* AA, *O. punctata* BB, *O. officinalis* CC, *O. minuta* BBCC) and the comparison with a cultivated species (*Oryza sativa* which genome is AA) (Dai et al., 2010). The phylogenetic analysis of *Pi2/9* gene family members from different *Oryza* species, demonstrated that genes within different species (i.e orthologs) are more similar to each other than to their homologues within the same species (parologs), suggesting that unequal recombination has not much impact on the evolution of the *Pi2/9* locus and that other mechanisms such as sequence mutation, gene translocation, inversion, and insertion of transposable elements has greatly contributed to the variation of genes in *Pi2/9* locus (Dai et al., 2010).

The B4 locus of common bean (*Phaseolus vulgaris*) was compared gene by gene with the sequenced portions of the three sequenced legume genomes, *Medicago trunculata* (*Mt*), *Lotus japonicas* (*Lj*) and *Glycine max* (*Gm*) (David et al., 2009). This comparison has revealed conserved microsynteny (conservation of local gene repertoire, order, and orientation) except for the CNL sequences, which appear to be completely absent in the corresponding regions of *Mt* and *Lj* and only a truncated CNL is present in syntenic regions of *Gm* (David et al., 2009). The authors suggested that CNL were inserted in the ancestor of the B4 locus after *Mt, Lj,* and *Pv* diverged but before the divergence of *Pv* and *Gm* through an ectopic recombination event between nonhomologous chromosomes. Phylogenetic analysis including those legume CNL sequences and all known B4-CNL sequences supported this hypothesis (David et al., 2009). Moreover, they presented additional evidence for the existence of ectopic recombination between nonhomologous chromosomes in common bean by means of the identification of a new 528-bp satellite DNA referred to as khipu, which is mostly associated with bean chromosome ends, in subtelomeric regions.

Genome wide analysis between all NB-LRR genes from two of closely related species, *A. thaliana* and *A. lyrata* has distinguished two evolutionary patterns: driven by positive or stabilizing selection (Chen et al., 2010). Most R-genes (50%) were evolving under strong positive selection characterized by high Ka/Ks ratios, frequent recombination, copy number variation, and extremely high sequence divergence between the two species. The stably selected R-genes (30%) had exactly the opposite four characters as the positively selected genes. The remaining R-genes (about 20%) were present in only one genome and absent from the other. A higher proportion of such genes were found to be part of TNL class (23.5%) compared to the non-TNL class (5.6%), suggesting different evolutionary patterns between these two groups. The diverse evolutionary patterns in R-genes should be the result of adaptation, which allows plants to cope with different types of pathogens.

PART III

1. Identification of candidate genes

Recent advance in plant genomics and the sequencing of both reference genomes and crop species have generate a lot of information that permits the rapid identification of candidate genes through bioinformatics analysis.

The strategies used for cloning genes of interest have been called 'Candidate gene approach' (CGA) (Byrne and McMullen 1996; Pflieger et al., 2001). The pre-requisite for a CGA is a repertoire of well characterized candidate genes (CGs) for a trait. The primary way to select functionally characterized CGs is to examine phenotypic, biochemical and physiological information on genes acting in the pathway of interest if this information is available (genomic sequence databases, literature, expression profiles, cellular localization of the corresponding protein, protein interactions, metabolic changes, mutant phenotypes and information from genetically modified organisms)(Bro and Nielsen 2004; Meyers et al., 2004).

Identification of CGs can be achieved using two different strategies: One approach use all information available from whole genome sequences in 'model' dicot and monocot species, to search genes of interest based on genomic synteny and on putative function of the genes present in that area. In this case the sequenced 'model' genome can be aligned with comparable marker sequences in a 'target' genome to deduce the putative CGs present in that genomic region (Salentijin et al., 2007).

The other approach involves the identification of CGs underlying a genomic region linked to a trait and required fine mapping studies as well as and genetic complementation studies (Salentijin et al., 2007). A schematically description of CGA is shown in Figure I.30.

1.1 Identification of CG genes based on genomic synteny

The first step in this approach is to identify the CGs in a 'model' and subsequently to identify the CGs in the 'target' crop by orthology. Within plant families the different genomes are often collinear which might offer the possibility to identify orthologous CGs on basis of syntenic genomic regions (Krutovsky et al., 2004). The essential genomic regions are predicted to be more conserved than the non-essential ones. The alignment of whole genomes therefore should reveal the more conserved regions that potentially will prove to be of functional importance (Chervitz et al., 1998).

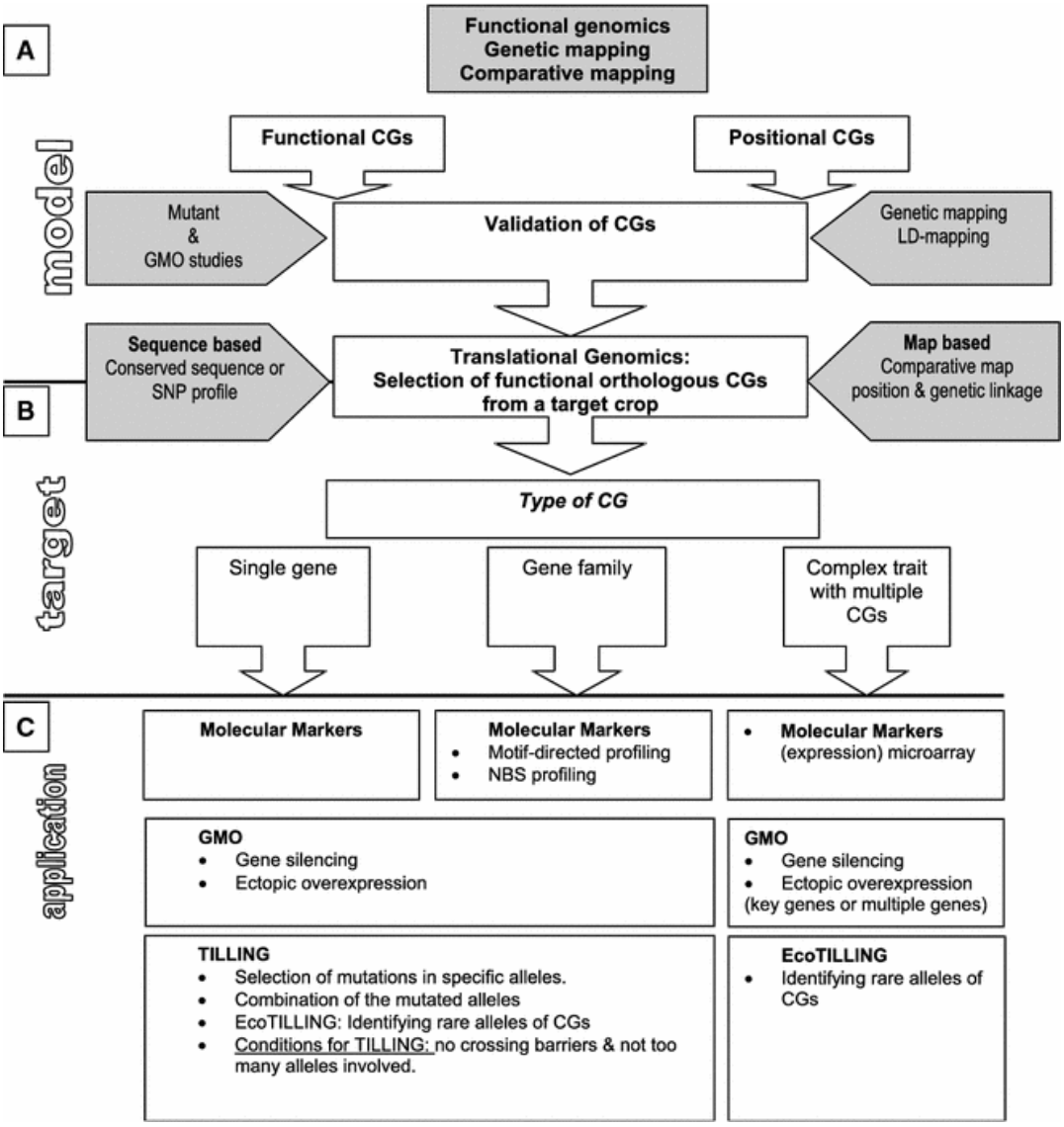

Figure I. 30 Route towards the application of candidate genes. From: Salentijn et al 2007.

However, extensive rearrangements and duplications taken place during evolution of some species can disturb the colinearity of the genomes and allow the loss of genes, consequently hampering the comparative mapping of CGs (Salentijin et al., 2007).

For example the gene *I2* from tomato which confers resistance to *Fusarium oxisporum* was compared with its syntenic region in potato, the locus *R3*. This comparison resulted in the *R3a* gene that confers resistance to *Phytophtora infestans* (Huang et al. 2005) but no to a *Fusarium.* That means, after the divergence of tomato and potato these loci may have evolved further resulting in a diversification of resistance genes based on co-evolution with the respective pathogens of tomato and

potato. This example of *R*-gene shows that comparative mapping of functionally validated R-genes may give a lead towards new candidate R-genes. But, even in case the complete genome sequence is available and QTLs for resistance are located, the identification of the specific R-gene copy within a cluster of RGAs may require laborious fine mapping and synteny studies (Salentijin et al., 2007).

RGAs are often located in clusters near to major QTL for resistance and can also be considered as CG (Pflieger et al., 2001; Kanazin et al., 1996; Mago et al., 1999; Pan et al., 2000; Ramalingam et al., 2003; van der Linden et al., 2004).

However, for crop species where no complete sequence is available, gene discovery relies on unassembled genome sequence data and expressed sequence tags (EST), SNP and simple sequence repeats (SSR) and molecular markers. Genetic mapping of these markers is required to determine their genomic location (Edwards and Batley 2010). Consequently, the actual involvement of the CG in most cases remains to be confirmed by genetic and physical mapping, positional cloning, expression analysis and genetic transformation experiments.

The coffee genome has not yet been completely sequence yet. The sequencing is now in progress (Argout et al., 2010). So in the present work the efforts were focus on the identification of candidate genes for resistance to coffee leaf rust using a positional cloning strategy.

1.2 Identification of CG using positional cloning strategy

The elaboration of genetic mapping is the first step toward the positional cloning. Genetic mapping places molecular genetic markers in linkage groups based on their co-segregation in a population. The genetic map predicts the linear arrangement of markers on a chromosome and maps are prepared by analyzing populations derived from crosses of genetically diverse parents, and estimating the recombination frequency between genetic loci (Edwards and Batley 2010). Different types of markers can be used for map construction such as SNPs, BACends, SSR etc.

In silico methods of SNP and SSR discovery are now being adopted, providing cheap and efficient methods for marker identification (Barker et al., 2003; Batley et al., 2003; Robinson et al., 2004; Jewell et al., 2006; Duran et al., 2009; Ganal et al., 2010). SNP involves finding differences between two sequences. Traditionally this has been performed through PCR amplification of genes⁄genomic regions of interest from multiple individuals selected to represent diversity in the species or population of interest, followed by either direct sequencing of these amplicons, or the more expensive method of cloning and sequencing. Sequences are then aligned and any polymorphisms identified (Edwards and Batley 2010).

The development of a physical map is the second step in positional cloning approach. Physical maps use all available techniques or informations to determinate the absolute physic position of a gene on a chromosome. Physical mapping is produced by ordering DNA clones from large-insert (typically BAC) libraries on the basis of a clone fingerprint pattern (Luo et al., 2003). Physical mapping can be used for positional cloning of genes/QTLs, studies on gene families, isolation of candidate genes and facilitate the analysis of genome structure, comparative genomics, and assembly of the entire genome sequence (Gu et al., 2010; Luo et al., 2010).

Strategies based on BAC fingerprints detect overlaps among BAC clones for the development of physical maps. Briefly, BAC clones are digested with restriction enzymes and the fragments are separated by electrophoresis, producing a pattern of bands. The overlap between adjacent clones is identified by pairwise comparison of band profiles and calculation of the proportion of shared bands (Meyers et al., 2004). The fingerprint profile generated by digestion, together with the BACend sequences and linkage map are used to organize the BAC clones forming contigs. The success of physical mapping depends on the fine genetic mapping of the locus of interest. Once the BAC clones covering the interval between the markers are found, the target gene can be identified by sequencing and by predicted gene mapping in relation to recombination events. The polymorphism analysis is conducted to evaluate the relation between polymorphism and variation at the character studied.

After candidate genes identification their function remains to be proved. The combination of all available functional information for a trait from physiological studies, microarray expression analysis and studies of gene function via transgenic functional validation or mutants are powerful approaches to determine gene function (Bro and Nielsen 2004). A summary of steps for positional cloning and gene validation is shown in Figure I.31.

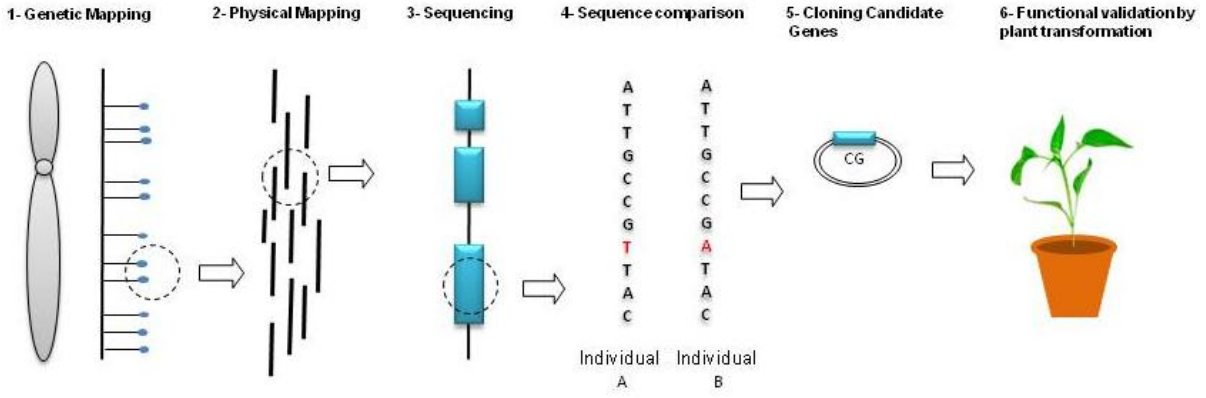

Figure I. 31 Steps of positional cloning. 1) Localization of region of interest by linkage analysis (molecular markers co-segregating with phenotype), 2) Assembly of genomic DNA clones (BACs) by BAC-end sequencing (contig formation), 3) sequencing of interest region and genes repertoire, 4) polymorphism studies and selection of candidate genes, 5) cloning of candidate genes at vectors for transformation, 6) plant transformation and phenotype analysis. From: Bouchez et al., 2006

2. Functional validation of CG

Recent advances in efficiency and accuracy of sequencing technologies have allowed the identification of several potential CG. The aim now is to determine the function of the predicted CG. Two main approaches are utilized to link genotype to phenotype are known as forward and reverse genetics. Both of these processes aim to determine the function of gene/genes through screening the phenotype or genotype of individual mutants to ultimately determine how it is controlled (Barkley and Wang, 2008).

The forward genetics (from phenotype to genotype) aims to identify the sequence change that underlies a specific mutant phenotype. The starting point is an already available or a specifically searched for and predicted phenotypic mutant of interest (Peters et al., 2003). On the other hand, in reverse genetics (from genotype to phenotype), the gene sequence is known and mutants are screened to identify individuals with structural alterations in the gene of interest (Alonso & Ecker, 2006). This approach is generally less time demanding than forward genetics and has been successfully used for functional genomics in many animal and plant species (Barkley and Wang, 2008).

Reverse genetic approach include *Agrobacterium*-mediated transformation, transposon tagging, RNAi (RNA interference) and point mutation using chemical mutagenesis (TILLING), each with its own strengths and weaknesses (Gilchrist, 2010).

The advantage and disadvantage of each reverse genetic approach has been recently reviewed by Barkley and Wang (2008) and are summarized below:

2.1 RNA interference

RNA interference (RNAi) has been widely employed to study gene function in plant and animal systems. RNAi is a post transcriptional gene silencing mechanism, which occurs through the recognition and degradation of double stranded RNA (dsRNA) into approximately 21-nucleotide RNAs known as 'small interfering RNAs' (siRNAs), by the Dicer enzyme present into cell. These siRNAs then provide specificity to the endonuclease-containing, RNA-induced silencing complex (RISC), which targets homologous RNAs for degradation preventing protein production. The dsRNAs are efficiently produced by intron-spliced hairpin transgenes (sense and antisense copies of the target gene separated by an intron). The dsRNAs can be delivered to plants in several ways including microprojectile bombardment with RNA (ihpRNA)-expressing vectors; infiltration of plant tissue with an *Agrobacterium* strain carrying a T-DNA expressing an ihpRNA transgene, virus induced gene silencing (VIGS), in which

the target sequence is integrated into viral sequences which are used to infect the plant, or are expressed from *Agrobacterium*-introduced transgenes, and by stable transformation with ihpRNA expressing transgenes (Matthew, 2004). The advantages in certain organisms include that the silencing is heritable and systemic (*i.e*. transmitted to the whole plant/organism). Disadvantages of this method include low throughput and efficiency of gene silencing and moreover this method can vary and be unpredictable. In loss-of-function approaches like RNAi or transposon tagging, tag sequences can be used to identify the genes disrupted by these elements. When the gene of interest has functionally redundant paralogous genes in the genome, no phenotype is expected in the loss-of-function lines (Curtis & Grossniklaus, 2003).

2.2 Chemical mutagenesis

TILLING, a high throughput mutation detection method, takes advantage of chemical mutagenesis to generate induced mutations in a population. Chemical mutagenesis has been used for a long time as a tool to obtain mutants for reverse genetic approach. Some of the chemical agents used to create mutants include ethylmethane sulfonate (EMS), N-ethyl-N-nitrosourea (ENU), N-nitroso-N-methylurea (NMU), and ionizing radiations. The advantages of chemical mutagens are that they tend to produce a relatively high density of random mutations throughout the genome including gene knockouts. Furthermore, induced point mutations generate a range of alleles for genetic analysis. Many plant species are well suited for this strategy because they can be self-fertilized and seeds can be stored for long periods of time and the use of chemical mutagens in diploid and polyploid plants produce a range of various mutations and a high density of mutations throughout the genome. Additionally, the creation of transgenic material is not required and the mutations are heritable in the successive generations. Lastly, unlike other reverse genetic approaches, chemical mutagenesis is applicable in most taxa.

The disadvantages of this approach include: i) when variation (polymorphism) in the trait of interest is high, efficiency of the technique decreased; the technique can generate false negative and false positive mutants; ii) it is an expensive technique; iii) the creation of a mutant population can be a challenge and sufficient time needs to be allocated for the development of a high quality population; iv) creating mutant populations for plants that are propagated vegetatively or have long generation times could also slow down progress of generating a mutant population; v) there is no clearly defined start or stop to your region of interest; vi) the interpretation is a challenge because the output gives only chromosome and position numbers necessitating the need to map a genome sequence in a separate step.

2.3 Transformation by *Agrobacterium*

Agrobacterium, a soil plant pathogenic bacterium, has become the most used method for the introduction of foreign genes into plant cells and the subsequent regeneration of transgenic plants. *A. tumefaciens* naturally infects the wound sites in dicotyledonous plant causing the formation of the crown gall tumors. *A. tumefaciens* has the exceptional ability to transfer a particular DNA segment (T-DNA) of the tumor-inducing (Ti) plasmid into the nucleus of infected cells where it is then stably integrated into the host genome and transcribed, causing the crown gall disease. Virulent strains of *A. tumefaciens* and *A. rhizogenes*, when interacting with susceptible dicotyledonous plant cells, induce diseases known as crow gall and hairy roots, respectively (Gelvin, 2003). The *A. tumefaciens* strain used for plant transformation carryies a modified Ti plasmid that was 'disarmed' by deletion of the tumor inducing genes. The genes to be introduced into the plant are cloned into a plant transformation vector that contains the essential parts of the T-DNA region of the disarmed plasmid, together with a selectable marker.

Transgenic plants can be engineered by *Agrobacterium* transformation to express the product of candidate genes. These approaches include phenotypic studies that can be made by generating gain-of-function or loss-of-function mutants. Advantages of this approach include generation of large mutation populations that can be created and stored before being exploited for variable aspects, as is currently available for the *Arabidopsis* model.

The advantages of gain-of-function approaches comparing to loss-of-function approaches for the characterization of gene functions include the abilities to (*a*) analyze individual gene family members, (*b*) characterize the function of genes from non model plants using a heterologous expression system, and (*c*) identify genes that confer stress tolerance to plants that result from the introduction of transgenes (Kondou et al., 2010).

A limitation of this method is sometimes an inefficient transgene expression. Another potential disadvantage is that in some plant species, transformation procedure has not been established or the efficiency is not enough to support functional gene analysis. Moreover for some species the transformation success can be genotype-dependent and hence the functional gene validation is restricted to few genotypes (Barkley and Wang, 2008).

3. Genetic transformation of woody plants

With the recent advances of massive sequencing technologies, an increasing number of groups around the world that are now interested in validating the gene function in different species including

woody plants. The first step for this consists in developing a good plant regeneration system for each species. However, many woody plants are still considered as recalcitrant to *in vitro* culture. A plant species or a developmental phase will be called recalcitrant if commonly used tissue-culture procedures fail to result in somatic embryogenesis (SE) or organogenesis. In other words, recalcitrance occurs if extensive manipulation of culture media by traditional means (high–low salt, organic and inorganic nitrogen levels and plant growth regulator levels among others) or by application of more recently discovered growth regulators (oligosaccharides, jasmonates and brassinosteroids) fail to produce the desired response (Bonga et al., 2010).

Woody plants are also harder to transform and require a long evaluation process after transformation. Consequently, most of reports so far published focused in developing or optimizing transformation protocols using marker genes (GUS or GFP) for example in grapevine (Dhekney et al., 2008), *Eucalyptus tereticornis* (Prakash & Gurumurthi, 2009) apricot (Petri et al., 2008) *Pinus* (Tereso et al., 2006), poplar (Cseke et al., 2007), rubber tree (Leclercq et al., 2010), *Leucaena leucocephala* (Jube & Borthakur, 2009), citrus (Dutt & Grosser, 2010).

Some traits of agronomical interest have also been genetically modified such as shortening of the juvenile phase in apple (Flachowsky et al., 2007; Trankner et al., 2010) and citrus (Peña et al., 2001), resistance to insect in *Pinus* (Grace et al., 2005), poplar (Rao et al., 2001) and coffee (Leroy et al., 2000); herbicide tolerance in *Pinus* (Bishop-Hurley et al., 2001; Parasharami et al., 2006) and coffee (Ribas et al., 2006), virus resistance in *Prunus* (Polak et al., 2008) and apple (Gambino et al., 2009), lignin biosynthesis in poplar (Halpin et al., 2007; Horvath et al., 2010), tolerance to abiotic stress in poplar (Strohm et al., 1999), *Pinus* (Tang *et al*. 2005, 2006) and grapevine (Zok et al., 2010), production of fruit seedless in citrus (Tan et al., 2009).

Improved resistance to diseases is one the most desirable character and has also been tested in woody plants. In apple, the expression of genes form *Vf* locus have been functionally analyzed (Malnoy et al., 2008). The *Vf* locus from *Malus floribunda* contains 4 genes (*Vfa1, Vfa2, Vfa3, and Vfa4*) and confers resistance to five races of the fungal *Venturia inaequalis* the causal agent of apple scab disease. To assess functionality, 3 of these genes were introduced into 2 susceptible cultivars of apple *via Agrobacterium*-mediated transformation. The *Vfa3* is a pseudogene and was not used. Transformed lines expressing *Vfa4* were found to be susceptible to apple scab, whereas those expressing either *Vfa1* or *Vfa2* exhibited partial resistance to apple scab. The transformation efficiency was 8-18% for Galaxy and 6-10% for McIntosh cultivars, respectively.

The Attacin E (antimicrobial proteins produced by *Hyalophora cecropia* moth in response to bacterial infection) has been used to produce apple trees resistant to fire blast caused by *E. amylovora* (Borjska-Wysocka et al., 2010). Stable expression of Attacin E has been observed during 12-year period increasing the resistance to fire blast and had no effect on tree and fruit morphology and quality.

The expression of Attacin A (isolated from *Tricloplusia ni*) has also been used in sweet orange to reduce severity of infection by *Xanthomonas citri* but no complete resistance was observed (Boscariol et al., 2006). Sarcotoxin from *Sarcophaga peregrina* also reduced severity of infection by *Xanthomonas citri* in *C. sinensis* cv. Pera (Bespalhok Filho et al., 2001).

The expression of PR-5 protein from tomato and bacterio-opsin from *Halobacterium halobium* has been used to reduce infection caused by *Phytophthora* spp in citrus (Fagoaga et al., 2001; Azevedo et al., 2006)**.** In both cases, an initial evaluation detected transgenic plants showing a significant reduction in lesion development as compared to the control non transformed plants.

The expression the hrpN gene that encodes a hairpin protein, which elicits the hypersensitive response and systemic acquired resistance in plants, was used to produce transgenic citrus (*Citrus sinensis*) hrpN lines. These lines showed reduction in susceptibility to citrus canker as compared with non-transgenic plants. One hrpN transgenic line exhibited normal vegetative development and displayed very high resistance to the pathogen, estimated up to 79% reduction in disease severity (Barbosa-Mendes et al., 2009). The hrpN has also been used to generate resistance to fire blight (*Erwinia amylovora*) in pear. Seventeen transgenic clones of the very susceptible cultivar "Passe Crassane" were evaluated. Most transgenic clones displayed significant reduction of susceptibility to fire blight *in vitro* when inoculated with *E. amylovora*, which was positively correlated to their degree of expression of the transgene hrpN (Malnoy et al., 2005).

The over-expression of endogenous *TcChi1* gene was studied in *Theobroma cacao* L. plants (Maximova et al., 2006). Chitinases are members of the pathogenesis-related protein family (PR-proteins), some of which have been shown to play a role in plant defense by degrading the chitin of fungal cell walls. The gene was under the control of a modified CaMV-35S promoter and was inserted into cacao genome by *Agrobacterium*-mediated transformation of somatic embryo cotyledons. Eight independent lines were generated. The transgene was expressed at varying levels in the different transgenic lines with up to a six fold increase of endochitinase activity compared to non-transgenic and transgenic control plants. The *in vivo* antifungal activity of the transgene against the foliar pathogen *Colletotrichum gloeosporioides* demonstrated that the TcChi1 transgenic cacao leaves significantly inhibited the growth of the fungus and the development of leaf necrosis compared to controls when leaves were wound inoculated with 5,000 spores. The results demonstrated the potential utility of the cacao chitinase gene for increasing fungal pathogen resistance in cacao.

Although many other examples could be cited, the production of transgenic plants is generally limited to few transformation events reflecting the low efficiency transformation for most woody plants.

3.1 Genetic transformation of coffee plants

Since the first explants of coffee were transformed (Barton et al., 1991; Spiral and Pétiard 1991), several studies have been conducted to improve the transformation efficiency in the two cultivate species *C. canephora* and *Coffee arabica* species. Different methods of transformation making use of reporter genes (GUS and GFP) and selective genes such as: antibiotics (hygromycin, kanamycin) or herbicides (chlorsulfuron, ammonium glufosinate) have been used to recover transformed coffee plants. However the transformation efficiency is still low. The results of these experiments are summarized in Table (I.11). This multiple protocols reflect how hard is to transform coffee and that none highly efficient method so far exist for *C. arabica* or *C. canephora*.

In spite all these efforts toward improving transformation efficiency for coffee the process still reminds tedious and not efficient. For this reason, only two examples of coffee transformation using genes of interest, both using *Agrobacterium*-mediated transformation exist. The first used the gain-of-function strategy to over express the *cry*1Ac gene from *Bacillus thuringiensis* that confer resistance to the leaf miner (*Perileucoptera coffeella*) in coffee plants (Leroy et al. 2000). Somatic embryos from *C. canephora* and *C. arabica* species were wounded and co-cultivated with *A. tumefaciens* carrying out a binary vector containing the *cry*1Ac gene. Transgenic coffee plants were proved to be resistant to leaf miner under greenhouse conditions and were also tested under field conditions in French Guyana during 4 years for field resistance (Perthuis et al., 2005) Among 54 different transformation events tested in this experiment, approximately 70% were proved to be resistant to leaf miner. The authors also observed that the transformed plants presented similar growth and development compared to non transformed control plants. It was the first time that an important agronomic trait was introduced into a coffee plant and field tested. However the transformation efficiency of coffee plants in this work was less than 1%.

The second example of transformation with an agronomically interesting gene used a loss-of function strategy to obtain transgenic coffee plants with suppressed caffeine synthesis (Ogita et al., 2003, 2004). The authors used RNA interference (RNAi) technology to inhibit the expression of the theobromine synthase gene (*CaMXMT1*). Two constructs containing either a short RNA fragment with 139 and 161 nucleotides or a long RNA fragment with two identical sequences of 332 nucleotides were prepared both containing a spacer fragment derived from *GUS* gene. The RNAi fragments were under the control of the CaMV35S promoter and nopaline synthase (NOS) terminator. Embryogenic tissues of *C. arabica* and somatic embryos of *C. arabica* and *C. canephora* were co-cultivated with *A. tumefaciens* carrying out RNAi vectors. Four stable transformed lines were obtained from *C. arabica.* Embryogenic tissues from 2 lines were used to analyze the efficiency of RNAi by estimating the levels of CaMXMT1 transcripts and the endogenous levels of theobromine and caffeine were determined

using HPLC. The theobromine contained in the two transgenic lines exhibited reduction rates of 85% and 65% while the caffeine content was reduced by 100% and 80% when compared to non transgenic coffee embryogenic tissues. *Coffea canephora* transgenic plantlets were produced from five independent lines using short RNAi (RNAi-S), five using long RNAi (RNAi-L) constructions and two using GFP (used as control for measures). Leaves of plants from two representative lines were selected and the transcript levels of the three methyltransferase genes were examined by RT-PCR. The results were similar to that observed for Arabica embryogenic tissue. The reduction in caffeine ranged from 40-70% depending on the transgenic line analyzed. This work demonstrated that the RNAi method was also effective *in planta,* although the suppression was not as complete as observed in embryogenic tissues of *C. arabica*.

The two examples cited above clearly demonstrated that transformation of coffee plants can be useful to validate the gene function. However the recovery of transgenic lines or plants in both examples was very low. For high-throughput gene validation purposes an optimized protocol for coffee transformation is required.

Table I. 11 Summary of *Coffee* sp transformation studies (Adaptated from: Ribas et al., 2006)

Coffea species	Explant used	Transformation method	Main results	Reference
C. arabica	Protoplast	*A. tumefaciens*	Transient *GUS* expression	Spiral and Pétiard (1991)
	Somatic embryos, cotyledons	*A. tumefaciens*	Kanamycin integration, no regeneration	Feng et al. (1992)
	Protoplasts	Eletroporation	Kanamycin integration, no regeneration	Barton et al. (1991)
	Somatic embryos - *arabica and arabusta*	*A. rhizogenes*	*GUS* integration, plant regeneration	Spiral and Pétiard (1993)
	Somatic embryos	*A. rhizogenes*, *A. tumefaciens*	Putative transformed callus, T-DNA amplification	Freire et al. (1994)
	Somatic embryos, leaves	Biolistic, plasmidial DNA	Transient *GUS* expression	Van Boxtel et al. (1995)
	Cotyledons, leaves	*A. rhizogenes*	Hairy-root plant regeneration	Sugiyama et al. (1995)
	Somatic embryos	*A. tumefaciens*	Cry1Ac, csr1-1 integration, plant regeneration	Leroy et al. (2000)
	Leaves, embryogenic tissue	Biolistic delivery	*GUS* expression	Rosillo et al. (2003)
	Embryogenic tissue, somatic embryo	Electroporation	*GUS* expression, secondary embryo formation	Da Silva & Yuffá, (2003)
	Embryogenic tissue, somatic embryos	*A. tumefaciens*	GFP expression, CaMxMT1 integration, transformed embryogenic tissue plant	Ogita et al. (2004)
	Embryogenic tissue	Biolistic delivery	*GUS* and *BAR* gene integration, plant regeneration	Cunha et al., (2004)
	Embryogenic tissue	*A. tumefaciens*	Antisense ACC and *BAR* gene integration, plant regeneration	Ribas et al. (2005c)
	Embryogenic tissue	Biolistic delivery	Kanamycin integration and *GUS* expression in F1 plants	Albuquerque et al. (2009)
	Hypocotyls from germinated zygotic embryos	*A. rhizogenes*	Production of transgenic roots and GUS expression	Alpizar et al. (2006)
C. canephora	Somatic embryos	*A. rhizogenes*	*GUS* integration, plant regeneration	Spiral et al. (1993)
	Somatic embryos	*A. rhizogenes*	*GUS* integration, plant regeneration	Spiral and Pétiard (1993)
	Somatic embryos	*A. tumefaciens*	Bt. integration, plant regeneration	Leroy et al. (1997)
	Embryogenic tissue	*A. tumefaciens*	*GUS* and HPT integration, plant regeneration	Hatanaka et al. (1999)
	Somatic embryos	*A. tumefaciens*	Cry1Ac, csr1-1 integration, plant regeneration, field tests	Leroy et al. (2000), Perthuis et al. (2005)
	Embryogenic tissue, somatic embryos	*A. tumefaciens*	GFP expression, CaMxMT1 integration, plant regeneration	Ogita et al. (2004)
	Embryogenic tissue	*A. tumefaciens*	*GUS* and *BAR* gene integration	Cruz et al., 2004
	Hypocotyl	*A. tumefaciens*	*GUS* and HPT integration, plant regeneration	Mishra and Sreenath (2004)
	Embryogenic tissue	Biolistic delivery	*GUS* and *BAR* gene integration, plant regeneration	Ribas et al., (2001, 2005a)
	Embryogenic tissue	*A. tumefaciens*	*GUS* and *BAR* gene integration, plant regeneration	Ribas et al., (2003, 2005c)
	Leaf explants, embryogenic tissue	*A. tumefaciens* assisted by vaccum infiltration	*DsRFP* (red fluorescent protein) and *NPT*II gene integration, plant regeneration	Canche-Moo et al., (2006)
	Secondary embryos	*A. rhizogenes*	Hygromicin (*hptII*) plant regeneration	Kumar et al., 2006
	Embryogenic cultures from hypocotyls and cotyledons	*A. tumefaciens*	GFP and hygromycin integration, plant regeneration	Kumar et al., 2010

Objectives

Coffee leaf rust (CLR) caused by the fungus *Hemileia vastatrix* is the main disease of coffee trees and cause important economical damage to coffee production. Currently, 9 major resistance factors (S_H1 to S_H9) single or in association appears conditioning resistance to coffee leaf rust in allotretraploid *C. arabica* species. The S_H3 resistance factor was identified in the Indian selections derived from tetraploid coffee S.26 and S.31 (*C. arabica* x *C. liberica* (Noronha-Wagner & Bettencourt, 1967; Bettencourt & Noronha-Wagner, 1971). Coffee plants harboring the S_H3 resistant factors have demonstrated agronomical acceptable durable resistance in field conditions (Prakash et al. 2005; Sera et al., 2007). Segregation analysis demonstrated that S_H3 is a single dominant gene (Prakash et al. 2004). The resistant genes (R genes) found in this locus belong to a multi genic family (Cenci et al., 2010). Until now none resistance gene against to CLR has been isolate. The characterization of resistance genes from S_H3 locus should contribute for conservation and valorization of the biodiversity in coffee trees.

In the last few years knowledge about organization and evolution of resistance genes in plants has greatly been improved. However less is now about these genes in perennial plants such as coffee. Divergent evolutionary pattern have been suggested between annual and perennial plants (Yang et al., 2008) but if this pattern could be generalized to all perennial plants is still unknown.

The function of predicted R genes must be validated. The selection of the approach should be based in the characteristic of the gene to be validated and the species. The most reliable way to determine the function of individual members of a gene family is engineering transgenic plants to express the product of candidate genes. Coffee leaf rust is a disease specific of coffee plants and in this case functional validations of candidate genes to CLR cannot be conducted in heterologous system such as *Arabidopsis* or tobacco for which genetic transformation is well established procedure. This phenomenon is known as restricted taxonomic functionality (Tai et al., 1999) where the failure of an *R* gene to confer resistance in a heterologous background may be due to the absence of other receptors, regulatory elements, or signaling or defensive genes required for the *R*-gene-mediated defense response. However, the regeneration and transformation of perennial plants such as coffee are not a well establish procedure. Although several studies have been achieved in coffee transformation (see table I.11) but transformation efficiency remainds low and. Moreover, none study was conducted to determine the target cell for coffee transformation.

The main objective of this thesis was to characterize the S_H3 locus that confers resistance to coffee leaf rust and to develop tools for functional gene validation in coffee trees.

The development of physical and genetic mapping of S_H3 locus is the start point toward the position cloning and is the subject of **chapter II**. To gain insight into genomic organization and evolution of the S_H3 locus, three coffee genomes were compared (C^a and E^a subgenomes of *C. arabica* and the C^c genome of *C. canephora*). R genes sequences present at this locus were analysed in order to point out forces driving evolution of R gene into S_H3 locus in **chapter III**.

A study focus on finding the target cells for coffee transformation and others parameters that influence coffee transformation efficiency is described in **chapter IV.**

In order to clone candidate genes to coffee leaf rust different approaches were essayed and are discussed in **chapter V.**

Finally, the the general discussion and the perspectives of this work is presented in **chapter VI.**

CHAPTER II

GENETIC AND PHYSICAL MAPPING OF THE S_H3 REGION THAT CONFERS RESISTANCE TO LEAF RUST IN COFFEE TREE (*Coffea arabica*)

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Genetic and physical mapping of the *S_H3* region that confers resistance to leaf rust in coffee tree (*Coffea arabica* L.)

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ABSTRACT

Resistance to coffee leaf rust is conferred by *S_H3*, a major dominant gene that has been introgressed from a wild coffee species *Coffea liberica* (genome L) into the allotetraploid cultivated species, *C. arabica* (genome C^aE^a). As the first step toward the map-based cloning of the *S_H3* gene, using a bacterial artificial chromosome (BAC) library, we describe the construction of a physical map in *C. arabica* spanning the resistance locus. This physical map consists in two homeologous BAC-contigs of 1170 and 1208 kb corresponding to the subgenomes C^a and E^a, respectively. Genetic analysis was performed using a single nucleotide polymorphism (SNP) detection assay based on Sanger sequencing of amplicons. The *C. liberica*-derived chromosome segment that carries the S_H3 resistance gene appeared to be introgressed on the sub-genome C^a. The position of the *S_H3* locus was delimited within an interval of 550 kb on the physical map. In addition, our results indicated a 6-fold reduction in recombination frequency in the introgressed S_H3 region compared to the orthologous region in *C. canephora*.

INTRODUCTION

Coffee is the world's most important agricultural commodity and is the main livelihood for more than 125 million people worldwide. Commercial coffee production relies mainly on two related species: *Coffea arabica* L. and *C. canephora* Pierre, which account for 65% and 35% of coffee production respectively (International Coffee Organization, http://www.ico.org). *C. arabica* L. (2n = 4x = 44), the only polyploid species in the *Coffea* genus, is an allotetraploid containing two diploid subgenomes, C^a and E^a, which originated from two different diploid species (2n = 2x = 22), *C. canephora* and *C. eugenioides*, respectively (Lashermes et al., 1999). Among the diseases affecting

cultivated coffee, coffee leaf rust (CLR) caused by the fungus *Hemileia vastatrix*, is one of the most serious. CLR is found in almost all coffee growing countries around the world and causes annual economic damage estimated at more than US$ 1 billion (van der Vossen 2005).

Efforts to obtain durable resistance to coffee leaf rust have a long history of initial successes followed by disappointments because of the appearance of new virulent races of the rust fungus (Kushalappa and Eskes, 1989; Prakash et al., 2005; Várzea and Marques, 2005). While the resistance genes identified in *C. arabica*, used either singly or in combination, have not provided durable resistance to most races of the rust fungus, the transfer into *C. arabica* of resistance genes from related diploid *Coffea* species can provide long-lived protection under field conditions (Srinivasan and Narasimhaswamy, 1975; Eskes et al., 1989). In particular, one resistance trait (i.e. S_H3 resistance factor) from *C. liberica* has been successfully introgressed into susceptible commercial *C. arabica* cultivars (Ramachandran and Srinivasan, 1979; Prakash et al. 2002). Classical genetic studies determined that the introgressed CLR from *C. liberica* is controlled by a single, dominant gene (S_H3), and amplified fragment length polymorphism (AFLP) markers have been identified that are linked to the resistance locus (Prakash et al., 2004). More recently, sequence-characterized molecular markers suitable for CLR marker-assisted selection and distributed within a distance of roughly 6 cM on the *C. liberica*-introgressed fragment have been reported (Mahé et al., 2008). While three markers were linked in repulsion with the S_H3 gene, seven markers were clustered in coupling around the S_H3 gene. These markers were used to screen a *C. arabica* BAC (Bacterial artificial chromosome) library and positive BAC clones were identified and roughly ordered without taking into account the allotetraploid structure of *C. arabica* (Mahé et al., 2007). Furthermore, using fluorescence *in situ* hybridization (FISH), the liberica-introgressed chromosome segment carrying the S_H3 resistance gene was located in *C. arabica* in a distal position on a chromosome belonging to the homeologous group 1 (Herrera et al., 2007).

Advances in molecular techniques and tools have facilitated the cloning of numerous plant disease resistance genes (R genes) in the past two decades (Martin et al., 2003). A method that has been shown to be successful for the isolation of genes of which only the phenotype and the map position are known is map-based cloning (Peters et al., 2003). However, map-based cloning is not always straightforward. A critical step is to construct a high resolution genetic map and to use this mapping information to isolate one or few genomic clones containing the gene of interest. For instance, difficulties can arise due to the high ratio of physical to genetic distance resulting from low levels of recombination or to the complex genome organization associated with plant polyploidy.

As the first step towards the map-based cloning of the S_H3 gene, we report here i) the generation in *C. arabica* of a physical map spanning the resistance locus ii) an evaluation of the recombination rate on the introgressed fragment carrying the S_H3 gene by comparison with *C. canephora*, and iii) the identification of a physical interval of 550 kb that contains the S_H3 locus.

MATERIALS AND METHODS

Plant material

The plant material analyzed in the present study consisted in individuals of both species *C. canephora* and *C. arabica*. Genetic mapping of *C. canephora* was performed in a mapping population consisting of 92 doubled haploids derived from the hybrid clone IF 200 (Lashermes et al. 2001). For *C. arabica*, the CLR susceptible line Matari and the CLR resistant line S.288 (S_H3-introgressed) were included in the analysis. In order to delimit the S_H3 region on the physical map, we used five *C. arabica* plants that were heterozygous for the liberica-introgressed chromosome segment carrying the S_H3 resistance gene and showed recombination between flanking markers of the S_H3 region. These five recombinant plants were selected based on previous molecular marker analysis (Mahé et al., 2008) in either a F_2 (Matari x S.288) population or a BC_2 population [(Matari x S.288) x Matari] x Matari. Their resistance reactions to CLR have been described in previous reports (Prakash et al., 2004; Mahé et al., 2008).

BAC fingerprinting and Southern hybridisation

Clones from a genomic DNA BAC library of the arabica cultivar IAPAR 59 (Noir et al. 2004) that have been previously end-sequenced and identified (Mahé et al., 2007) as positive in filter hybridization experiments with genetic marker probes surrounding the S_H3 gene were selected for the construction of BAC contigs (Table II.1). BAC DNA was extracted using an alkaline lysis procedure (Sambrook et al., 1989). To estimate the size of the BAC inserts, we applied *Not*I-digested DNAs on pulse field gel-electrophoresis (PFGE) as described in Noir et al. (2004).

Agarose gel-based BAC DNA fingerprinting was performed as described by Marra et al. (1997) with some modifications. DNAs of the selected BAC clones were digested with either the restriction enzyme *Hind*III or *Eco*RI. DNA fragments were separated into a 1% agarose gel by electrophoresis at 80 V in circulating 1x TAE buffer at 4°C for 16 h. A mixture of marker size DNA (Raoul, MPbiomedicals) was loaded into every fifth lane. The gel was stained in 1 l of a 1: 20000 dilution of Vistra Green (Molecular Probe, USA) in 1x TAE for 30 min. Gel images were captured using a scanner (Typhoon 9400, Amersham Biosciences) and exported as tagged image files. Fingerprints were edited using the Image 3.10 module of the FingerPrinted Contig (FPC) package (Sanger centre, USA). BAC contigs were assembled using FPC (version 7.2). Sulston scores of 1×10^{-10} and 1×10^{-13} were tested using the stringent conditions of tolerance 5.

Following agarose gel electrophoresis, the digested BAC DNAs were also transferred to Hybond-N^+ nylon membranes for Southern hybridization as described in Noir et al. (2004). Probes were labelled with [^{32}P]-dCTP according to the manufacturer's recommendations (Megaprime DNA Labelling Systems kit, Amersham) and hybridisation was carried out as described by Sambrook et al. (1989).

Table II. 1 Reference and size of BAC clones selected for contig construction around the *S_H3* locus.

BAC clone	Size (kb)	Probe [a]
144-13G	145	BEs2
60-3C	115	BEs2
82-6F	210	BEs2
140-17D	175	BEs2
131-11D	111	BEs2
55-21G	205	BEsnh3
55-13B	210	BEsnh3, BEs3
34-20L	240	BEsnh3, BEs3
64-1L	200	BEs3
41-23C	110	BEs6
56-20G	148	BEs6
121-12I	180	BEs6
124-12K	177	BEs6
68-6F	96	BEs6
82-8K	200	BEs6
136-1N	219	BEs6
131-1P	145	BEsnh4
140-24N	150	BEsnh4
52-11O	218	BEsnh4, BEs19
70-19D	202	BEs19
38-13H	200	BEs19, BEs12
45-4E	275	BEs19, BEs12
34-11L	135	BEs19, BEs12
80-9A	187	BEs19, BEs12
143-8C	200	BEs19, BEs12
48-21O	194	BEs12

[a] Probe from the *S_H3* region showing positive hybridisation in BAC filter screening experiments (Mahé et al., 2007)

Genetic mapping

For each plant of the mapping population and the parental accessions, genomic DNA was extracted from 50-100 mg of lyophilized leaves by the method of Diniz et al. (2005). Amplification conditions using specific primer pairs (either SSR or SCAR), radioactive labeling and polyacrylamide gel electrophoresis were as reported elsewhere (Mahé et al., 2008). The markers were revealed on an Amersham Phosphor Screen and screened after 24 hr with a Typhoon 9700 (Amersham Biosciences) to obtain digital images. Linkage analysis and map construction were performed using the computer program MAPMAKER version 3.0b (Lander et al., 1987). The canephora doubled haploid population was treated as a backcross population with an expected segregation of 1:1 for the two alternative alleles present in clone IF 200. Recombination frequencies were converted into map distances or centimorgans (cM) using the Kosambi function.

Sequence comparison and SNP detection assay

Specific primer pairs targeting BAC-end sequences were used to amplify DNA from BACs, the two Arabica parental lines, Matari and S.288, selected recombinant plants and one doubled haploid (i.e. accession DH200-94) derived from the Canephora IF 200 clone. These PCR products were directly sequenced from both sides using the gold standard ABI 3730xl DNA Analyzer platform (Cogenics, France) based on the dideoxy chain termination DNA sequencing method (Sanger et al. 1977). The termination peak profiles from the different samples were aligned and the single nucleotide polymorphisms (SNP) detected using the Staden Package (Staden et al., 2000).

RESULTS

Construction of BAC contigs in the S_H3 region

A total of 26 *C. arabica* BAC clones containing genetic markers surrounding the S_H3 locus were selected for contig construction (Table II.1). The insert sizes of the isolated BAC clones were estimated to be from 50 to 275 (average of 173.5) kb by PFGE of *Not*I-digested fragments. The 26 BAC clones were fingerprinted using restriction enzymes. On average, 30 restriction fragments per

clone and per enzyme, ranging from 1 kb to 50 kb nucleotides, were obtained and included in the FPC analysis. While a unique contig of 26 clones was obtained at a Sulston score of 1 x 10^{-10}, increasing the Sulston scores to the more stringent condition of 1 x 10^{-13} resulted in a total of six contigs comprising two to six BAC clones.

Table II. 2 Comparison for 4 BAC-end regions of sequences derived from the two BAC-contigs C^a and E^a with sequences amplified from an accession of *C. canephora* (DH200-94, genome C).

BAC-ends references	GenBank acc. numbers	Primer sequences (5'>3')	% substitutions (number of aligned nucleotides)	
			C^a vs C	E^a vs C
34-20L-f	ED989446	For: tccatccatgctcactttga Rev: ttgcaagaaagcagcatctc	0.00 (551)	2.37 (548)
124-12K-r	ED989455	For: acagttgaaaaacggatgc Rev: ccttgctgctttcttgaacc	0.00 (291)	2.06 (291)
41-23C-r	ED989447	For: ttggccttcactccagtctt Rev: cggttgaggatttggtccta	0.00 (363)	0.83 (363)
136-1N-f	ED989457	For: tcaatgagcagtccatcagc Rev: ttccatcttgtttgggaagc	0.66 (304)	2.63 (304)

BAC-end (BE) marker probes from the different BAC contigs were generated using specific PCR primer combinations and used to perform Southern hybridisation on the blots of either *Hin*dIII or *Eco*RI-digested BAC DNAs. The orientation and overlapping of these clones were identified. In addition, simple and clear polymorphism was observed between BAC clones in six BE probes (64-1L-f, 136-1N-r, 45-4E-r, 143-8C-r, 45-4E-f, 124-12K-r) enabling, for each BE, the classification of positive BAC clones in two groups that correspond putatively to the two Arabica subgenomes (C^a and E^a)(Figure II.1).

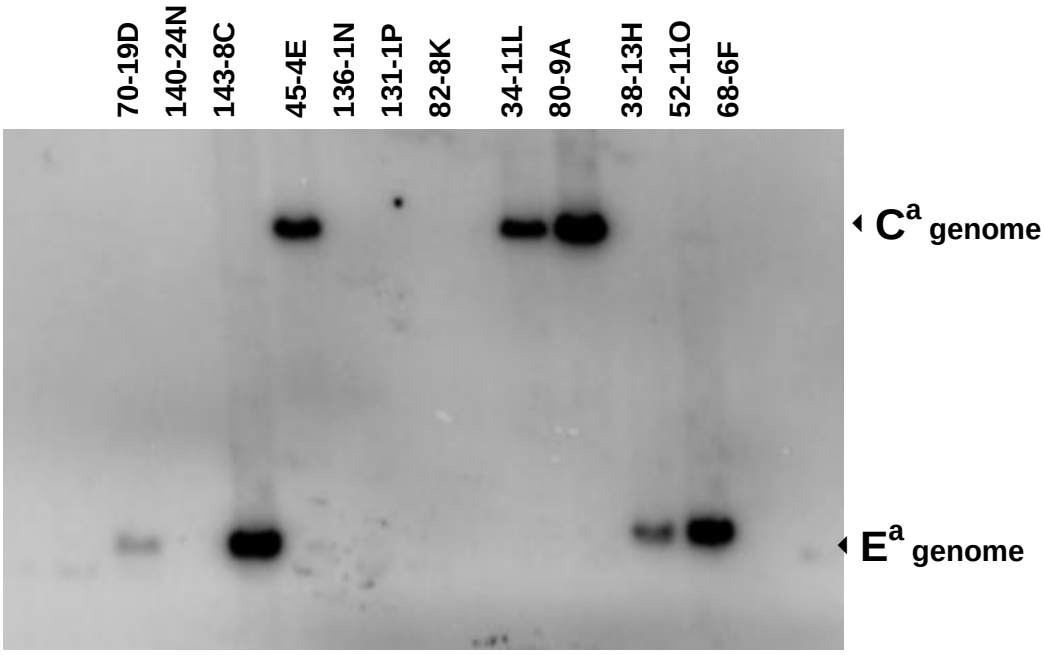


Figure II. 1 Southern hybridisation of *Hin*dIII-digested DNA from BAC clones of the S_H3 region by a BAC-end marker probe (143-8C-r). Based on the polymorphism revealed, the positive clones were classified in two groups that putatively correspond to the two Arabica subgenomes.

By combining the results of the restriction fragment analysis and Southern hybridisation, BAC clones were assembled into two contigs that represent the homeologous chromosome segments around the S_H3 locus (Figure II. 2). BAC clone position and overlapping were consistent using the different approaches. The putative C^a contig contained 12 BAC clones and was estimated to span over 1170 kb. Regarding the putative chromosome segments E^a, 14 BAC clones were assembled and the overall physical map was estimated to span over 1208 kb.

To ascertain the assignment of the BAC-contigs to the C^a or the E^a sub-genomes, regions corresponding to 4 BAC-ends were sequenced in BACs belonging to the two contigs and in accession DH200-94 of *C. canephora*, and compared (Table II.2). For all four regions, the sequences (ranging from 291 to 551 bp) derived from the BACs belonging to one contig (named C^a) appeared notably more similar to the sequences obtained in *C. canephora* (C genome) than the sequences derived from the second BAC contig (named E^a). On average, the level of substitution between the contig C^a and the C genome was 0.17% while 1.97% of substitution was observed between the contig E^a and the C genome.

Comparative genetic mapping of the *S_H3* region in *C. arabica* versus *C. canephora*

A total of 13 sequence-characterized genetic markers associated with the *S_H3* region, as previously determined (Mahé et al., 2008) based on the liberica-introgressed Arabica F2 (Matari x S.288) population analysis, were selected for genetic linkage analysis in *C. canephora*. The ability of these sequence-specific primers to reveal polymorphism in the *C. canephora* doubled haploid (DH) mapping population was tested. The eight markers showing polymorphism were genotyped in the 92 doubled haploids and the *C. canephora* linkage map was updated. All analyzed markers mapped to the linkage group 1 of the *C. canephora* map. The genetic maps of the *S_H3* region as determined in the liberica-introgressed F2 Arabica population were then compared with the corresponding region (linkage group 1) in *C. canephora* (Figure II.3). In the liberica-introgressed Arabica F_2 population, two linkage groups related to the *S_H3* region were identified, one being the introgressed fragment carrying the *S_H3* gene (i.e. markers in coupling) while the other represented the corresponding fragment inherited from the non-introgressed line (i.e. markers in repulsion). A total of seven markers were common to the Arabica and Canephora maps. While these markers mapped within an interval of 39.3 cM in *C. canephora*, they resulted in two small linkage groups in the liberica-introgressed F_2 Arabica population of 5.9 (introgressed segment) and 5.6 cM, respectively. This represents a roughly 6-fold difference in recombination frequency in the same genetic interval between these two populations. Furthermore, differences in genetic distances between shared markers were observed along the whole segment.

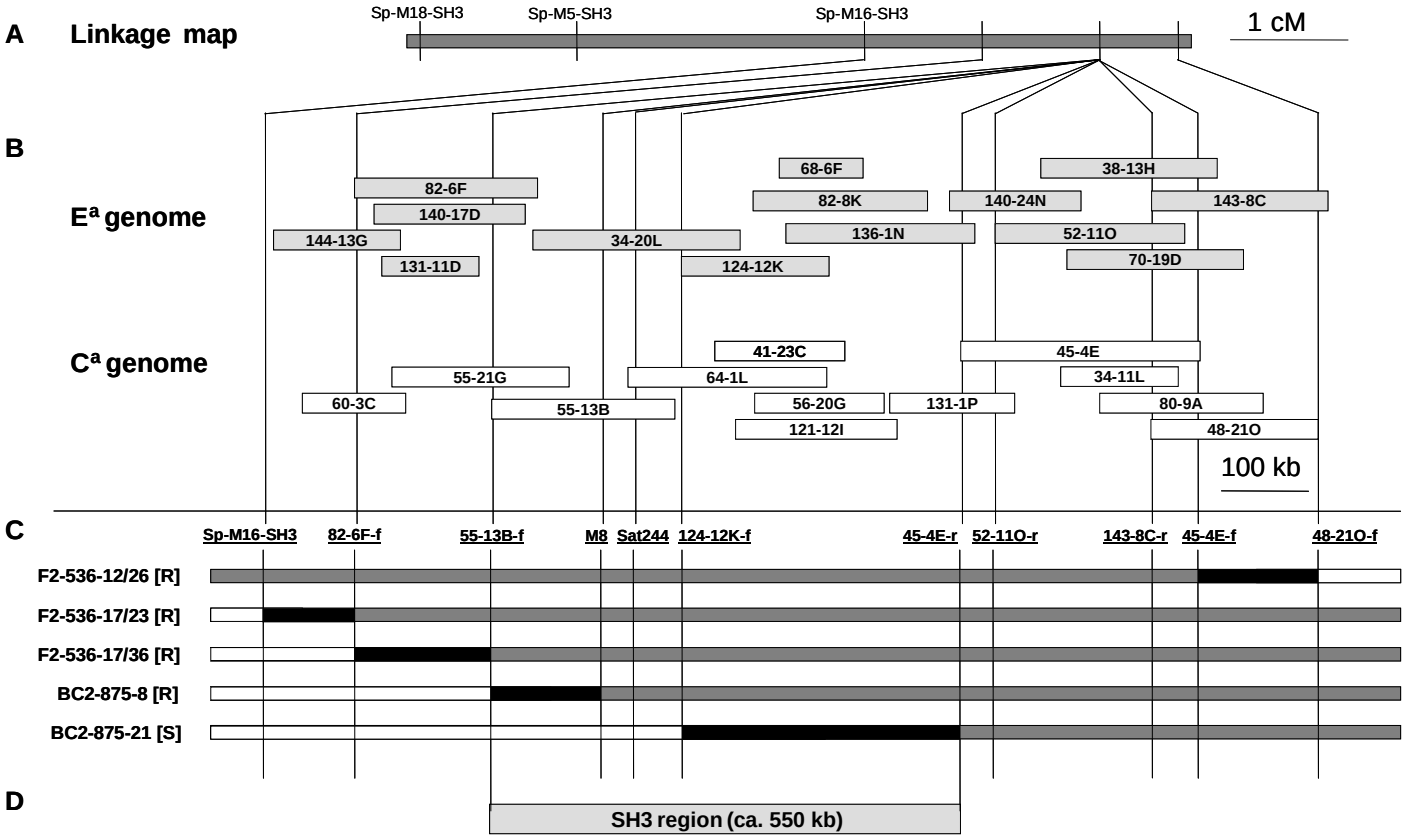


Figure II. 2 BAC clone contig around the S_H3 gene locus, and the S_H3 region on the physical map. A The linkage map of the introgressed chromosome segment carrying the S_H3 gene based on the F_2 (Matari x S.288) population analysis. B Overlapping *C. arabica* BAC clones forming the contig in the S_H3 related region. Boxes of BAC clones belonging to the E^a and C^a sub-genomes are in grey and white, respectively. C The BAC-derived markers and the graphical genotypes of three F_2 and two BC_2 recombinant plants. The grey bars represent chromosome segments derived from S.288 (introgressed rust-resistant line) while the hachured bars indicate the marker-intervals enclosing the recombination breakpoints. Rust resistance reaction is presented in brackets following the plant code: R resistant, S susceptible. D The present maximum limits of the region containing the S_H3 locus on the physical map.

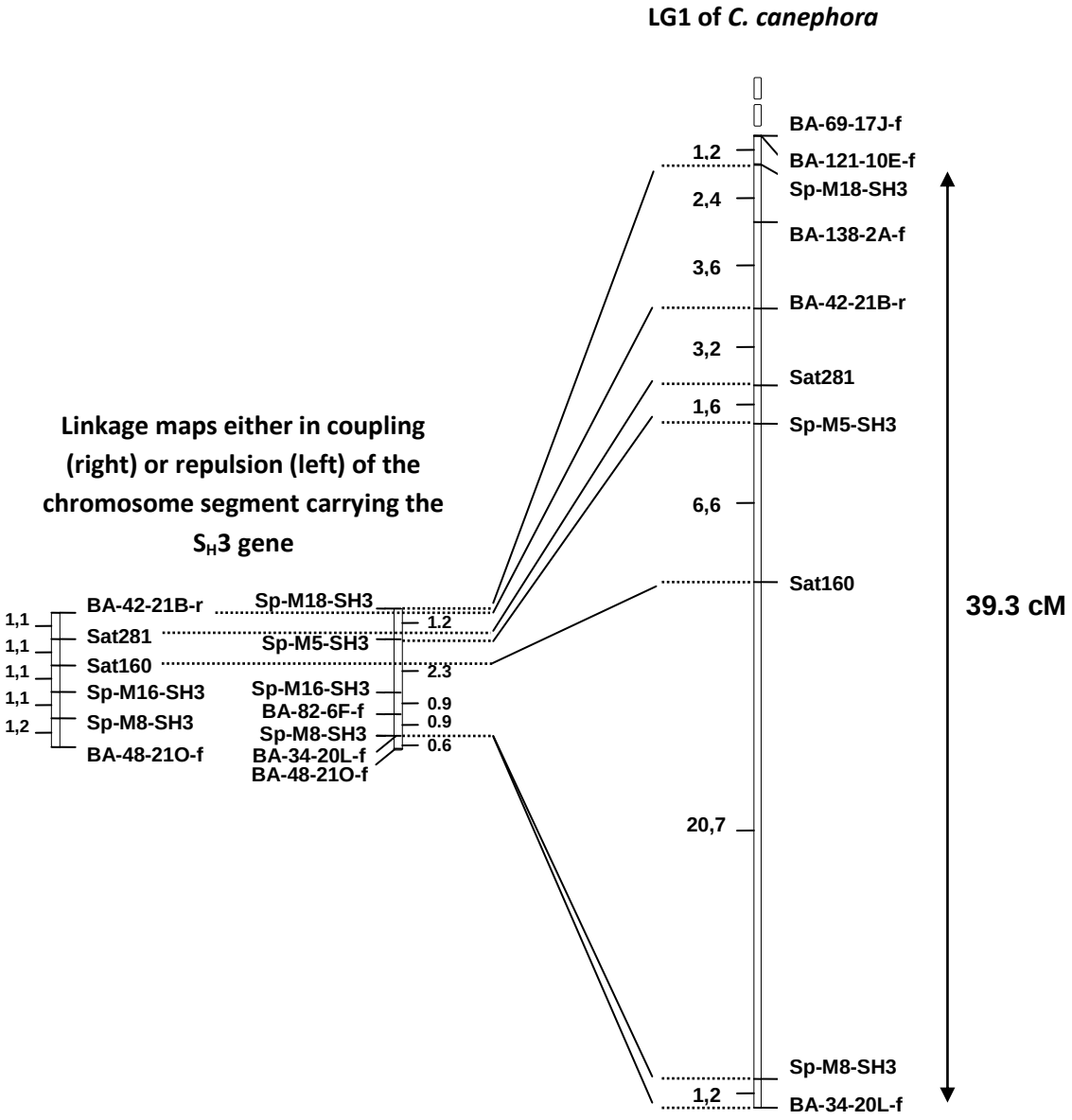


Figure II.3. Comparison of genetic maps of the S_H3 region determined in *C. arabica* based on results of analysis of the liberica-introgressed F_2 (Matari x S.288) population (Mahé et al. 2008) with the corresponding region (linkage group 1) in *C. canephora* (Lashermes et al. 2001). Shared markers are linked by lines. The estimated map distances are indicated in cM (Kosambi function).

Identification of a physical interval containing the *S_H3* resistance locus

Heterozygous plants exhibiting recombination events in the *liberica*-introgressed chromosome segment carrying the *S_H3* resistance gene were rescreened with BAC-end-derived genetic markers obtained as a result of physical mapping. In total, five recombinant plants were analyzed, three plants from the F_2 (Matari x S.288) population and two plants from the BC_2 population [(Matari x S.288) x Matari] x Matari. PCR products obtained from the selected recombinant plants and control samples were directly sequenced using the dideoxy chain termination DNA sequencing method (Sanger method). The termination peak profiles from different samples were aligned and the patterns compared to detect SNPs (Figure II.4). On the 16 BAC-end-derived amplicons, eight gave unreadable sequences (multiple fragments) and were discarded. The remaining eight exhibited at least one map-informative SNP (i.e. SNP between introgressed and non-introgressed parents). Assignments of single SNP or patterns of SNPs to either the subgenomes C^a and E^a or to the liberica-introgressed segment (L) were inferred by comparing the BAC-end-derived sequences and the sequences amplified in the parental liberica-introgressed line S.288 and in the *C. canephora* accession 200-94. For the eight loci, the sequences of the subgenome C^a were not present in line S.288 while the sequences attributed to the liberica-introgressed segment (L) were identified. The genotype at each locus was then determined in recombinant plants by analyzing the relative ratio between the different haplotypes observed in a given plant (Figure II.4). The recombination breakpoint in the five recombinant plants was localized in intervals between markers (Figure II.2). The markers were named according to the BAC-end clones from which they were derived. Taking into account the CLR evaluation of recombinant plants, the *S_H3* resistance locus was located between markers 55-13B-f and 45-4E-r, in an interval predicted to be less than 550 kb.

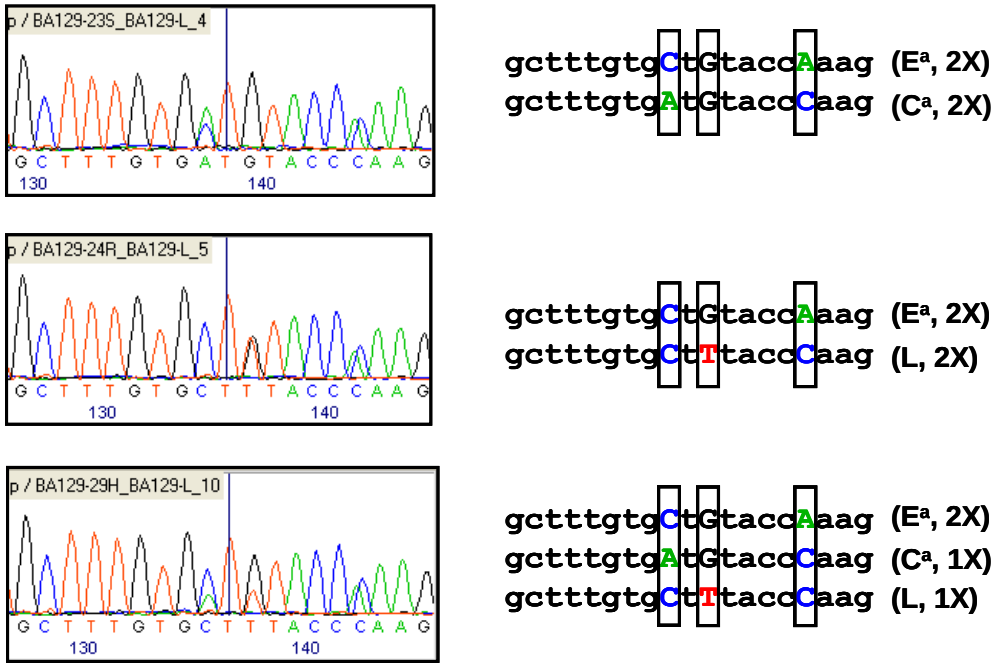


Figure II.4. Example of SNP detection assay based on Sanger direct sequencing of amplicons (BAC-end-derived marker 45-4E-f) from plants of the F_2 (Matari x S.288) population. The termination peak profiles and the deduced sequence combinations are presented for three contrasted plant samples (two parental homozygous and one heterozygous). The detected SNPs, indicated by boxes, were assigned to the *C. arabica* sub-genomes (C^a or E^a) or the liberica-introgressed chromosome segment (L). The C^a sequence was determined on a *C. canephora* genotype, the E^a sequence was deduced by subtraction on the sequence of the non-introgressed arabica parent and the *C. liberica* sequence (L) was deduced from the sequence of the introgressed parent. Identified haplotypes with their dosage are given in parenthesis.

DISCUSSION

Development of a physical map of the S_H3 region

In an effort to clone a CLR gene using a map-based cloning approach, we constructed a physical map of the S_H3 region in C. arabica. In line with the allotetraploid structure of *C. arabica*, this physical map consists in two homeologous contigs of 1170 and 1208 kb corresponding to the

subgenomes Ca and Ea, respectively. As a consequence of the high sequence similarity between the two subgenomes, analysis of restriction fragment-based BAC fingerprints was not straightforward. So, when BACs shared restriction fragments, it was not always possible to determine if these common restriction fragments resulted from overlapping or homeology. In contrast, Southern blot hybridizations of the digested BAC DNAs allowed clear classification of positive BAC clones according to the subgenome. Combining restriction fragment analysis with Southern blot hybridization data to construct a physical map thus appeared to be very efficient in a polyploid species such as *C. arabica.*

Efficient SNP detection method based on dideoxy-terminated sequencing

Marker development in polyploids can be time-consuming and often represents a major challenge in breeding programs and genetic research. Single-nucleotide polymorphism (SNP) markers have become the technology of choice for almost all organisms because of their wide distribution in genomes and their suitability for high multiplex detection systems (Rafalski et al. 2002). However, in the case of polyploids, difficulties arise due the presence of homeologous sequences that may confuse SNP detection, and when a SNP is detected, the genome in which it is located cannot be readily deduced. The SNP detection assay based on Sanger sequencing of amplicons that was applied in the present study appears to be very efficient and appropriate for polyploids. The SNPs are detected directly based on the analysis of the termination peak profiles and there is no need to develop and validate genome-specific primers or probes. This approach is highly suitable for map saturation of specific regions because virtually all the SNP in not repeated regions can be detected. The high success rate in the detection of informative SNP is probably due to the divergence between the regions involved (i.e. nucleotide polymorphism) between Ca and Ea sub-genomes and an introgressed segment from the *C. liberica* genome). In an intraspecific context, the success of this approach will depend on the genetic divergence of the parental lines.

The introgression from *C. liberica* into *C. arabica* is associated with repressed recombination

Analyses of the S.228 line that carries the CLR S_H3 resistance gene that has been transferred from C. liberica revealed an introgression of the sub-genome C^a. In addition, our results indicated a 6-fold reduction in recombination frequency over the entire S_H3 region in the Arabica F_2 (Matari x S.288) population compared to the orthologous region in *C. canephora*. This reduction is most likely

the consequence of the introgressed origin (from *C. liberica*) of this region in the S.288 line. Reduced recombination frequencies have been frequently observed around disease resistance genes in plants including barley, grape, poplar, wheat and tomato, when resistance has been introduced via an introgressed region from a related species (Ganal et al. 1996; Wei et al. 1999; Stirling et al. 2001; Neu et al. 2002; Barker et al. 2005). In contrast, the recombination rate estimated for a canephora-introgressed chromosome segment of *C. arabica* was recently reported to be very similar to the recombination frequency reported in *C. canephora* (Lashermes et al. 2010). This contrasted observation likely reflects differences in genetic divergence between chromosomes belonging to the C^a and L genomes and the close relatedness between the C (i.e. *C. canephora*) and the C^a sub-genome.

An average physical/genetic relationship of 570 kb/cM was reported for *C. canephora* (Lashermes et al 2001). Assuming a similar average ratio in C. arabica, a 1000-kb contig would represent approximately 1.8 cM of genetic distance. It was therefore interesting to note that plants exhibiting recombination events in the *S_H3 liberica*-introgressed chromosome segment were identified without difficulty although a reduction in recombination frequency was observed. These data strongly suggest that recombination in the *S_H3* region is much higher than the average rate for the *C. canephora* genome. The frequency of recombination varies substantially throughout the genome (Nachman 2002). For instance, while the heterochromatic regions near centromeres and telomeres of individual chromosomes are known to show considerable recombination suppression, the subtelomeric regions, by contrast, have functions that greatly enhance recombination (Drouaud et al 2006). The *S_H3* locus is in the subtelomeric region of canephora chromosome 1 (Herrera et al. 2007). Thus, the suggested enhanced recombination is consistent with the subtelomeric location of *S_H3*.

Towards cloning of *S_H3*

The results described here provide a basis to investigate the structure of the *S_H3* resistance locus. The establishment in *C. arabica* of a physical interval of 550 kb that contains the *S_H3* locus represents a significant step. This interval could be easily narrowed down by analysing additional BAC-derived genetic markers on the same set of recombinant plants. However, from now on, BAC clones representing a minimal tiling path could be selected and complete DNA sequencing generated using the latest generation of sequencing technologies that make reading DNA fast, cheap and widely accessible (Hert et al 2008). Once the resistance locus is deciphered, a further step required to identify the *S_H3* gene(s), would be to work with a CLR resistant genotype to isolate homologous sequence(s) and validate the identified candidate sequence(s) by complementation tests using *Agrobacterium tumefaciens* mediated genetic transformation (Etienne et al. 2008).

CHAPTER III

ORGANIZATION AND MOLECULAR EVOLUTION OF A DISEASE RESISTANCE GENE CLUSTER IN COFFEE TREES

This chapter was written as an article and submitted to BMC Genomics

Organization and molecular evolution of a disease-resistance gene cluster in coffee trees

Alessandra F. Ribas, Alberto Cenci, Marie-Christine Combes, Hervé Etienne and Philippe Lashermes

ABSTRACT

Background: Most disease-resistance (R) genes in plants encode NBS-LRR proteins and belong to one of the largest and most variable gene families among plant genomes. However, the specific evolutionary routes of NBS-LRR encoding genes remain elusive. Recently in coffee tree (Coffea arabica), a region spanning the *S_H3* locus that confers resistance to coffee leaf rust, one of the most serious coffee diseases, was identified and characterized. Using comparative sequence analysis, the purpose of the present study was to gain insight into the genomic organization and evolution of the *S_H3* locus.

Results: Sequence analysis of the *S_H3* region in three coffee genomes, E^a and C^a subgenomes from the allotetraploid *C. arabica* and C^c genome from the diploid *C. canephora*, revealed the presence of 5, 3 and 4 R genes in E^a, C^a, and C^c genomes, respectively. All these R-gene sequences appeared to be members of a CC-NBS-LRR (CNL) gene family that was only found at the *S_H3* locus in C. arabica. Furthermore, while homologs were found in several dicots, comparative genomic analysis failed to find any CNL R-gene in the orthologous regions of other eudicot species. The orthology relationship among the -CNL copies in the three analyzed genomes was determined and the duplication/deletion events that shaped the *S_H3* locus were traced back. Gene conversion events were detected between paralogs in all three genomes and also between the two sub-genomes of *C. arabica*. Significant positive selection was detected in the solvent-exposed residue of the *S_H3*-CNL copies.

Conclusion: The ancestral *S_H3*-CNL copy was inserted in the *S_H3* locus after the divergence between Solanales and Rubiales lineages. Moreover, the origin of most of the *S_H3*-CNL copies predates the divergence between *Coffea* species. The *S_H3*-CNL family appeared to evolve following the birth-and-death model, since duplications and deletions were inferred in the evolution of the S_H3 locus. Gene conversion between paralog members, inter-subgenome sequence exchanges and positive selection appear to be the major forces acting on the evolution of *S_H3*-CNL in coffee trees.

BACKGROUND

In their natural environment, plants encounter a vast array of pathogenic microorganisms such as viruses, bacteria, oomycetes, fungi and nematodes. To defend themselves against infection by these pathogens, plants employ a network of intertwined mechanisms. One such line of defense is based on dominant disease resistance (R) genes that mediate resistance to pathogens possessing corresponding avirulence (Avr) genes (Jones and Dangl, 2006). The largest class of known R genes includes those that encode the nucleotide binding site (NBS) and the leucine-rich repeat (LRR) domains. The deduced NBS-LRR proteins can be subdivided in classes based on their amino-terminal features (Dangl and Jones, 2001; Meyers et al., 1999). The most frequent classes possess a TIR domain with similarity to either the intracellular signaling domains of Drosophila Toll and the mammalian Interleukin-1 Receptor or a CC domain (coiled-coil) in the N-terminal and are named TNL (TIR-NBS-LRR) and CNL (CC-NBS-LRR), respectively (Dangl and Jones, 2001; Jones and Dangl, 2006; Martin et al., 2003). Each domain of NBS-LRR protein is predicted to have a specific function. The NBS domain is suggested to have NTP-hydrolyzing activity (ATPase or GTPase, etc), regulating signal transduction through conformational changes (Leipe et al., 2004; Martin et al., 2003). The LRR domain contains tandemly arrayed repeats in the carboxy-terminal region of R-genes and its predicted biochemical function is to mediate protein-protein interaction. It was hypothesized and experimentally confirmed that the LRR domain is involved in the specific recognition of pathogen effectors (Ellis et al., 2000b; Hammond-Kosack and Jones, 1997; Jones and Jones, 1997; Van der Hoorn et al., 2001). Both TIR and CC domains are assumed to be involved in protein-protein interactions and signal transduction (Jebanathirajah et al., 2002; McDowell and Woffenden, 2003).

Genes encoding NBS-LRR protein represent one of the largest and most variable gene families found in plants, with most plant genomes containing several hundred family members. NBS-LRR genes are unevenly distributed in plant genomes and are mainly organized in multi-gene clusters (Ameline-Torregrosa et al., 2008; Hulbert et al., 2001; Meyers et al., 1999; Richly et al., 2002; Yang et al., 2008a; Zhou et al., 2004). Furthermore, results of nucleotide polymorphism analyses demonstrated extremely high levels of inter and intraspecific variation of NBS-LRR genes, which presumably evolved rapidly in response to changes in pathogen populations (Meyers et al., 2005; Yang et al., 2008a; Yang et al., 2008b). The clustered distribution of R-genes is assumed to provide a reservoir of genetic variation from which new pathogen specificity can evolve via gene duplication, unequal crossing-over, ectopic recombination or diversifying selection (Michelmore and Meyers, 1998). However, the specific evolutionary routes of NBS-LRR encoding genes remain elusive. Several comparative sequence analyses of R-gene clusters have been performed across haplotypes or related genomes in different plant species including *Arabidopsis* (Meyers et al., 2003b; Noel, 1999), wild

potato (Kuang et al., 2005a), tomato (Kruijt, 2004; Parniske and Jones, 1999; Seah et al., 2007a), *Brassicaceae* (Xiao et al., 2004), wheat (Wicker et al., 2007), rice (Dai et al., 2010) soybean (Innes et al., 2008) and common bean (David et al., 2009a). Available data suggest that different R genes can follow strikingly different evolutionary trajectories. Kuang et al. (Kuang et al., 2004; Kuang et al., 2005b) divided NBS-LRR-genes into two evolutionary categories: Type I includes genes whose evolution is accelerated by frequent sequence exchange among paralogs. Consequently, their sequences have chimeric structure and a clear allelic/orthologous relationship between different genotypes cannot be easily established. Type II includes slowly evolving genes whose sequence mainly evolves through the accumulation of amino acid substitution. Orthology relationships are highly conserved among accessions (Kuang et al., 2008).

The evolutionary rate of each domain of individual NBS-LRR-encoding genes has been shown to be heterogeneous (Kuang et al., 2004). The NBS domain appears to be subject to purifying selection, whereas the LRR region tends to be highly variable (McHale et al., 2006). Nucleotide polymorphisms found in the LRR region of *R* genes have been shown to be responsible for pathogen specificity (Ellis et al., 2000b). In particular, codons encoding putative solvent-exposed residues in the LRR domain are hypervariable among different R proteins and show significantly elevated ratios of non-synonymous to synonymous substitutions, suggesting that the LRR domain is subject to positive selection for amino acid diversification (Ellis et al., 1999b; McDowell et al., 1998; Michelmore and Meyers, 1998; Parniske, 1997; Shen et al., 2002; Yahiaoui et al., 2006).

Coffee is one of the world's most important agricultural commodities and is the main livelihood of more than 80 million people worldwide. Although the *Coffea* subgenus *Coffea* includes more than 95 species (Davis et al., 2006), commercial coffee production relies mainly on two related species: *Coffea arabica* L. and *C. canephora* Pierre, which account for 65% and 35% of world coffee production, respectively (International Coffee Organization, http://www.ico.org). *C. arabica* L. (2n = 4x = 44), the only polyploid species in the *Coffea* genus, is an allotetraploid containing two diploid subgenomes, C^a and E^a, which originated from two different diploid species (2n = 2x = 22), *C. canephora* and *C. eugenioides*, respectively (Lashermes et al., 1999). While diversification in the *Coffea* subgenus *Coffea* probably occurred in the second half of the Middle Pleistocene (450,000–100,000 years BP), it is most likely that the allopolyploid speciation of *C. arabica* took place in relatively recent times i.e. from historical times to 50,000 years ago (Anthony et al., 2010; Lashermes et al., 1999).

Among the diseases affecting cultivated coffee, coffee leaf rust, caused by the obligate parasitic fungus *Hemileia vastatrix* Berk. & Br. (Uredinales), is one of the most serious diseases and greatly limits Arabica coffee production in almost all coffee growing countries around the world. Therefore, the development of coffee varieties resistant to coffee leaf rust has been a breeding

objective of the highest priority in many countries (van der Vossen, 2005). A number of resistance genes to coffee leaf rust have been identified in the cultivated or wild *Coffea* gene pool. In particular, one resistance gene (i.e. S_H3 resistance factor) has been successfully introgressed from *C. liberica* into agronomically important Arabica cultivars. In the last few years, the genetic and physical maps of the S_H3 locus were completed (Lashermes et al., 2010; Mahe et al., 2007; Prakash et al., 2004). Furthermore, using fluorescence *in situ* hybridization in *C. arabica*, the S_H3 locus was located in a distal position on a chromosome belonging to the homeologous group 1 (Herrera et al., 2007). Recently, a region of 800 kb spanning the S_H3 locus was sequenced and annotated (Cenci et al., 2010). Tandem arrays of CNL R genes were identified suggesting that the S_H3 locus corresponds to a complex multi-gene cluster.

The purpose of the present study was to gain insight into the genomic organization and evolution of the S_H3 R gene cluster in coffee. The agronomic importance of this locus as well as the recent origin and the perennial characteristic of coffee species make these objectives especially appealing. Sequences of the S_H3 region in three different genomes, the C^a and E^a subgenomes of *C. arabica* and the C^c genome of *C. canephora* were analyzed to investigate the genomic organization and evolution of the S_H3 locus. In addition, we performed comparative analyses of the identified NBS-LRR encoding sequences to identify the forces that drive evolution in the S_H3 R gene cluster. Our results highlight the importance of intra and inter subgenomic gene conversion as an important evolutionary mechanism for the evolution of disease resistance genes.

RESULTS

Organization of the S_H3 R gene cluster

The sequences of a total of 13 BAC clones spanning the S_H3 locus (Figure III.1) in three coffee genomes (i.e. E^a and C^a sub-genome from *C. arabica* and C^c from *C. canephora*), were examined for the presence of R-genes using the previously determined annotation (Cenci et al., 2010). Depending on the genome concerned (E^a, C^a, C^c), a total of 5, 3 and 4 R-genes were identified, respectively. These sequences shared more than 95% of identity. According to Chen et al. (Chen et al., 2010), a R gene family is defined when 60% identity is shared by members. The R-gene sequences detected in S_H3 were therefore further considered as members of a unique R-gene coffee family. BLAST analysis of the non-redundant database (All non-redundant GenBank CDS translations + RefSeq Proteins + PDB + SwissProt + PIR + PRF) revealed high similarity with several R-genes belonging to the CNL class. While the highest identity was observed with the hypothetical CNL R

protein in *Vitis vinifera* (36% identity) in *Ricinus communis* and *Glycine max* (35%), the most similar functionally characterized protein was the RPP8 gene from *Arabidopsis thaliana* that confers resistance to *Peronospora parasitica* (McDowell et al., 1998) and shares 32% of identity and more than 50% of similarity.

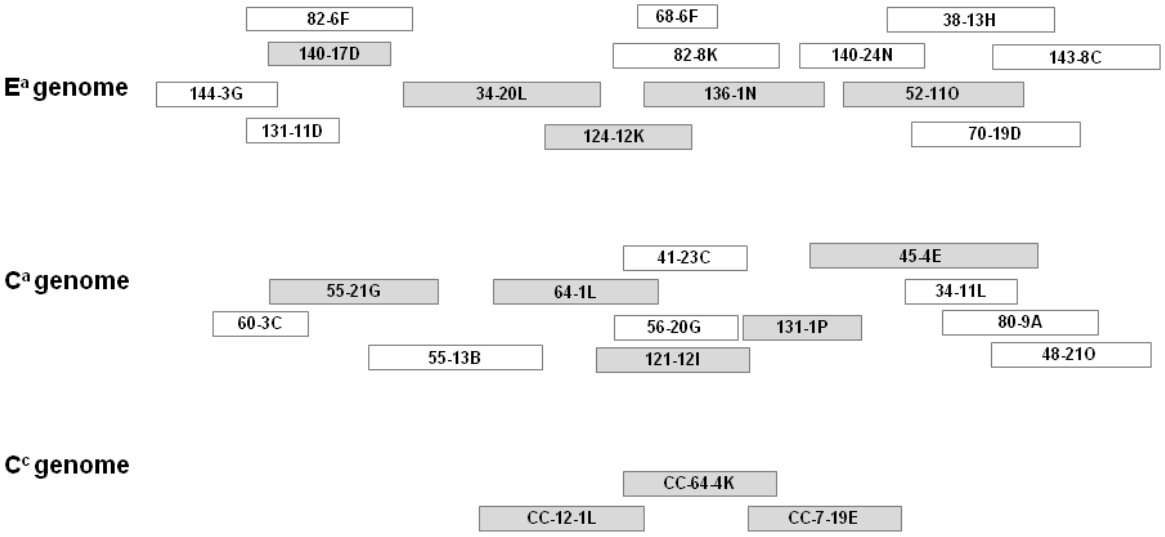


Figure III. 1 BAC clone contigs spanning the S_H3 locus. BAC clones spanning the S_H3 locus as previously contigued (Cenci et al., 2010). Sequenced BACs from the three genomes are indicated by gray boxes.

The CNL-like sequences identified in the S_H3 R-gene cluster were distributed in two regions separated by more than 160 kb (Figure III. 2). In the first region (hereafter called region A) two or three copies were found in the same orientation. The other region (B) contained 1 or 2 copies repeated in tandem. Region A and B had opposing coding orientations. Orthology relationship among R-genes was established by comparing flanking sequences and each member was identified by the group letter followed by a number. A homeologous non-reciprocal transposition event (HNRT) occurred between the two genomes of *C. arabica* and involved a region of around 50 kb that includes the A1 and A2 members (unpublished data).

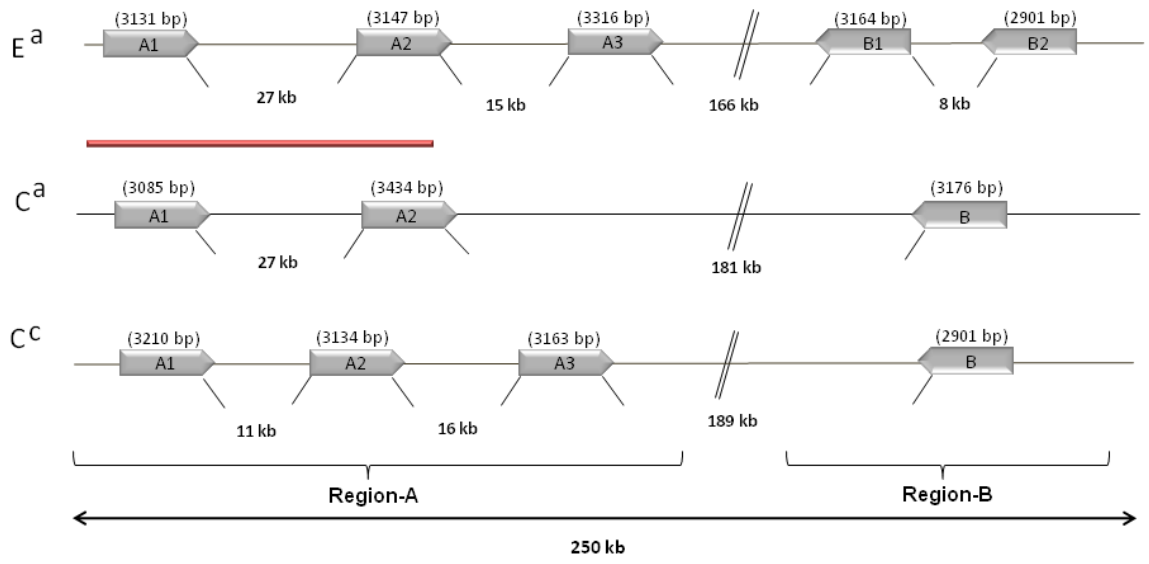


Figure III. 2 Organization of S_H3-CNL members in three coffee genomes: E^a and C^a represent the sub-genomes from *C. arabica*, and C^c the genome from *C. canephora*. The red bar represents a 50 kb region where a homeologous non-reciprocal transposition event (HNRT) occurred between the two genomes of *C. arabica* (unpublished data).

To test for the presence of a possible additional copy of the S_H3-CNL in the Arabica coffee genome, Southern blot analysis was performed using a specific probe corresponding to a conserved part of the NBS region (Figure III. 3). Whatever the restriction enzyme used, only a limited number of hybridization bands was detected. Based on the restriction profiles predicted from sequence analysis of *C. arabica* cv. IAPAR-59 BAC, it was possible to assign all the bands to one of the eight members (five in the E^a genome and three in the C^a genome) present at the S_H3 locus. No additional band was detected, suggesting that this family is only present at the S_H3 locus in *C. arabica* cv. IAPAR-59.

The presence and number of members of this gene family in a panel of diploid coffee species were investigated by Southern blot analysis (Figure III. 4). While the S_H3-CNL family was always present, the number of members ranged from three to eight depending on the species. Intra-specific variability was also observed for different accessions of *C. canephora* and *C. eugenioides*.

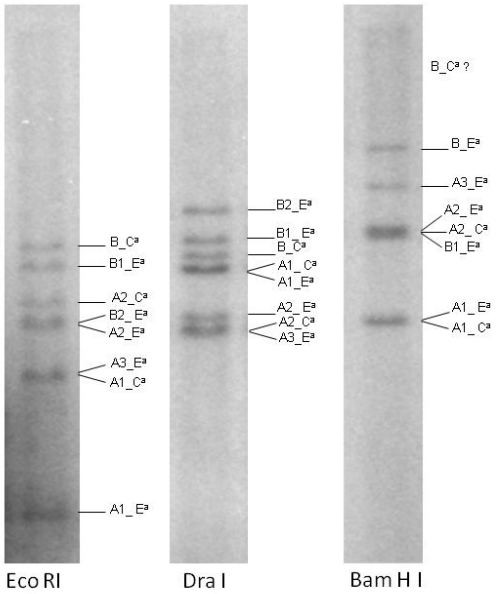


Figure III. 3 Southern blot hybridization of genomic DNA of *C. arabica*: DNA from the IAPAR-59 accession was digested with *Eco*RI, *Dra*I and *Bam*HI enzymes. The probe corresponded to the part of the NBS region that is highlighted in gray in figure 2.

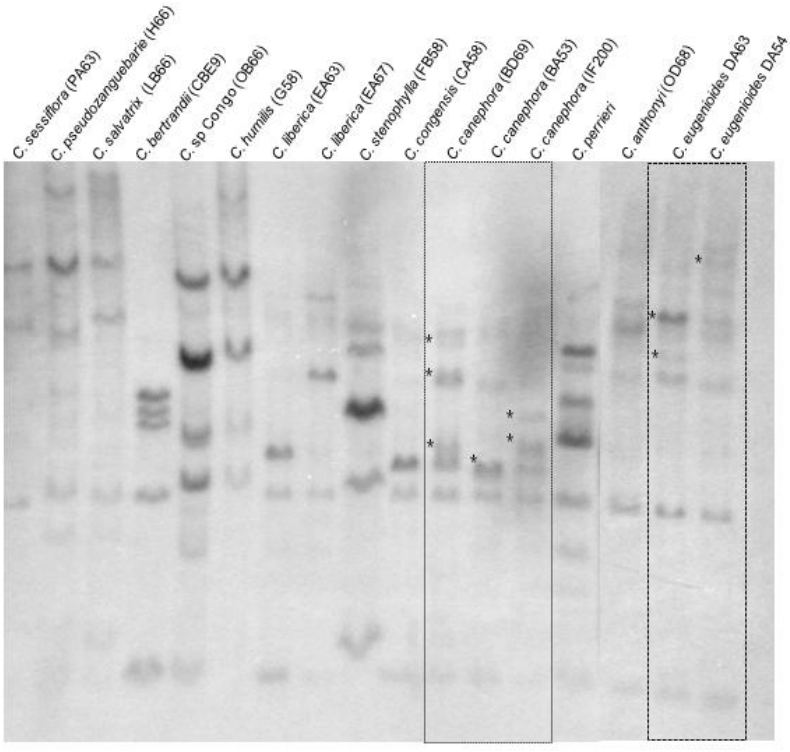


Figure III. 4 Southern blot hybridization of genomic DNA from diploid coffee species: *Eco*RI restricted DNA from diploid coffee species was probed with a NBS domain fragment. The stars indicate different band size among accessions of *C. canephora* and *C. eugenioides*.

Origin and evolution of the S_H3 R gene cluster

To investigate the origin of the S_H3-CNL genes present at locus S_H3 we performed comparative analysis of the available sequences of three *Coffea* genomes and among the S_H3-CNL copies including their flanking regions. Since members of the S_H3-CNL family were found to be collinear in the comparisons of the three *Coffea* genomes (Figure III. 5A), we concluded that the observed organization of this locus predates the divergence between *C. eugenioides* and *C. canephora* lineages. The most parsimonious scenario for the evolution of this locus is illustrated in Figure III.5 B. Two tandem duplications and several deletions shaped region A, whereas a distant duplication/insertion event gave birth to the S_H3-CNL member(s) in region B.

Locus S_H3 was compared with the putative orthologous region in the tomato genome (*Solanum lycopersicum*) which is, to date, the closest species to *Coffea* for which whole genome sequence is available (http://solgenomics.net). Micro-synteny was found between the coffee S_H3 locus and two tomato genomic regions which shared 53.2 and 23.4% of the *Coffea* genes, respectively (data not shown), but no CNL genes were found in these regions of the tomato genome.

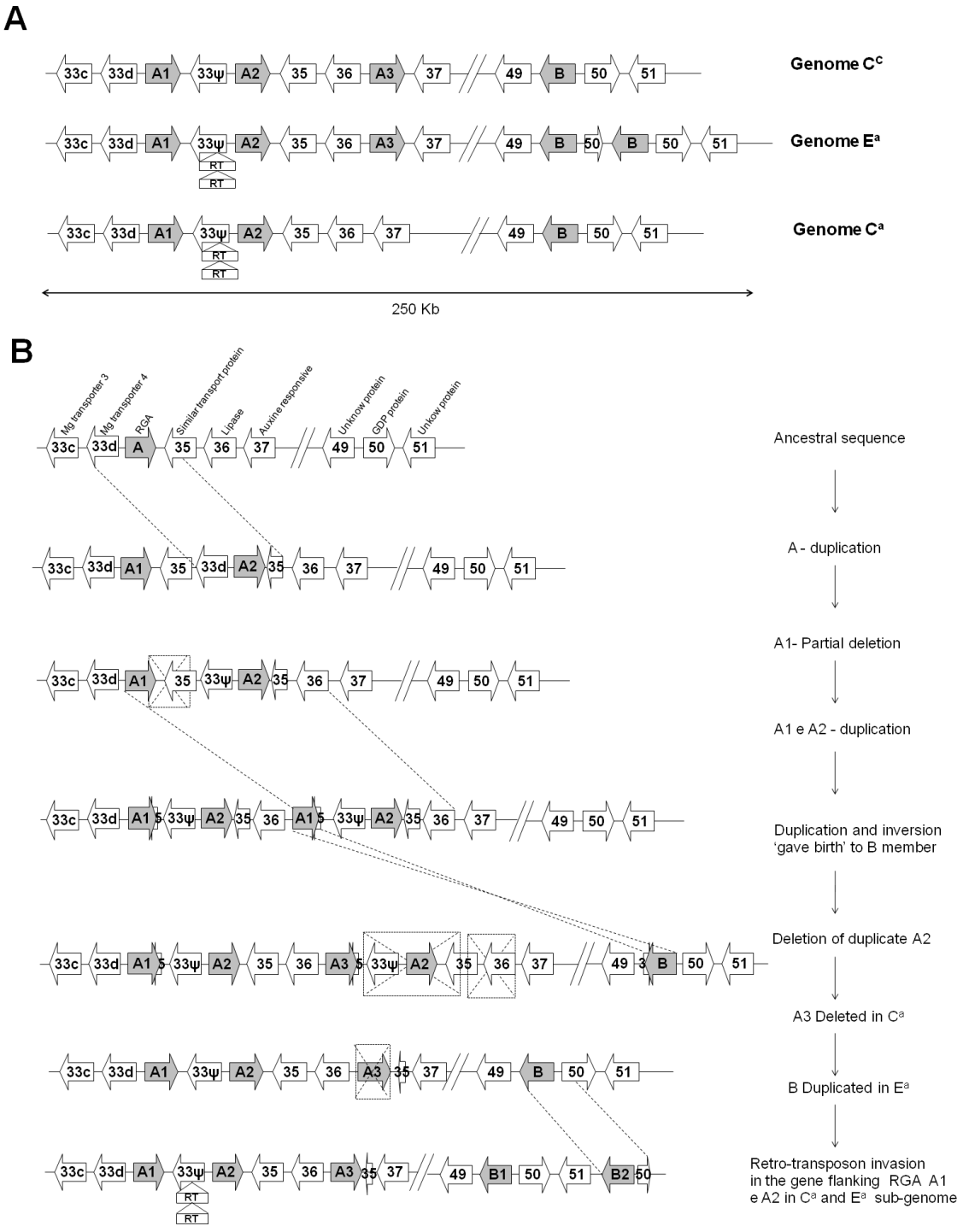


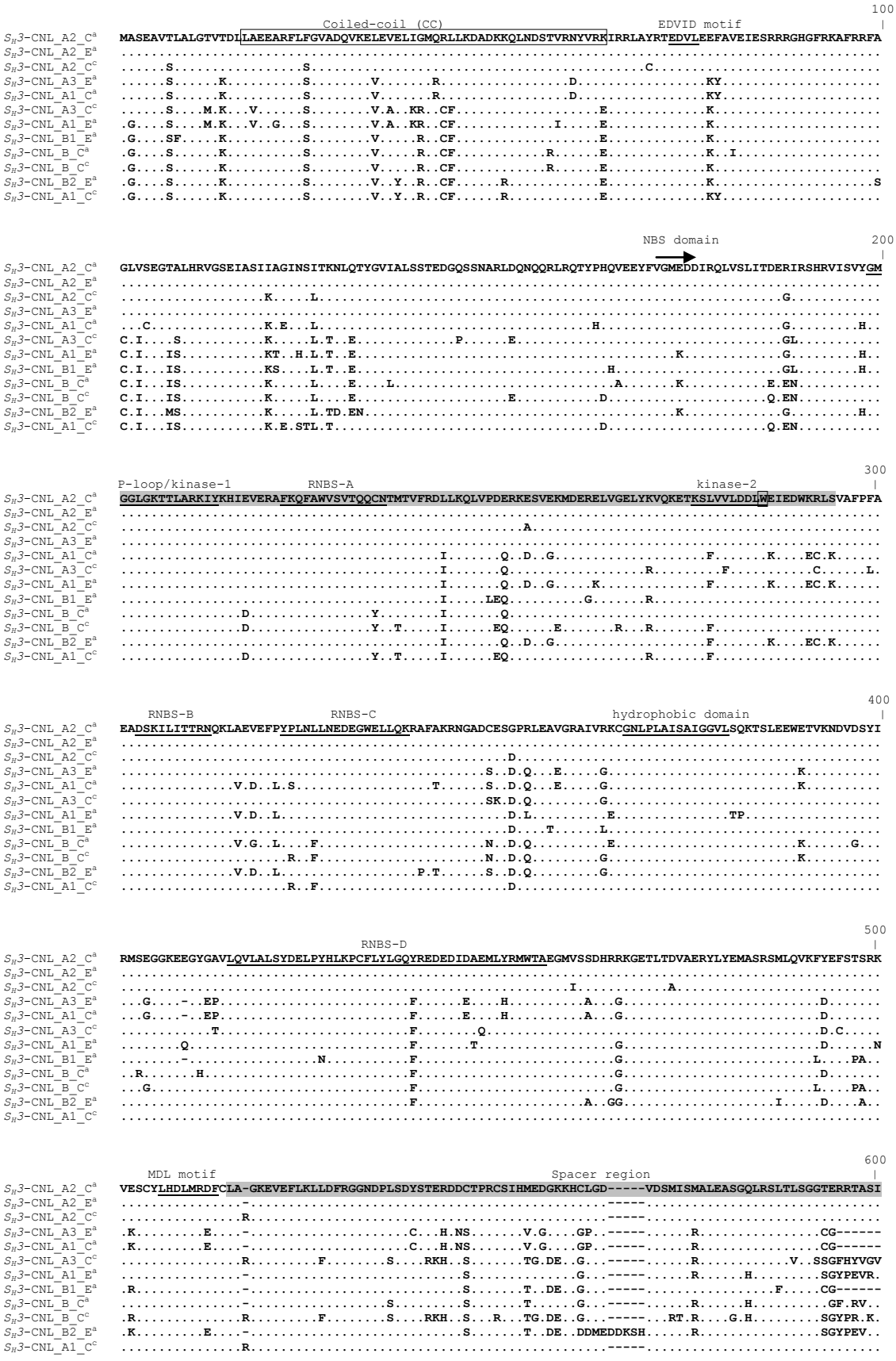
Figure III. 5 Evolution of the S_H3 locus in coffee species: A. Current organization of the S_H3 locus in *Coffea canephora* (C^c) and *C. arabica* (sub-genome E^a and sub-genome C^a). B - A model of the evolution of locus S_H3 in coffee plants involving genome expansion and retraction by gene duplication and deletions. Gray arrows indicate members of the S_H3 family. Open arrows indicate other non-R genes flanking R genes in the locus as numbered in [48]. Short arrows indicate truncated versions of corresponding genes

Sequence characterization of the S_H3-CNL family

The coding sequence of all S_H3-CNL members is composed of two exons separated by an intron ranging from 157 to 272 nucleotides in length. The first exon spanned 1042 nt while the second exon extended from 1703 to 2003 nt (Table III.1). The protein sequence extended from 915 to 1015 aa (Table III. 1). The protein sequence alignment of the identified 12 S_H3-CNL members (eight from *C. arabica* and four from *C. canephora*) is shown in Figure III.6. S_H3-CNL_A2_C[a] was chosen as query to annotate protein domains. BLASTp analysis against the Pfam database predicted a NBS domain between positions 173 and 465 aa, while analysis of the Conserved Domain Database predicted the beginning of the LRR region at position 625 aa of the query protein. COILS analysis revealed a coiled-coil region located between position 17 and 56 aa, confirming that this family belongs to the CC sub-family of NBS-LRR genes (or non-TIR sub-family). The LRR region of all genes consists of 12 repeats ranging from 23 to 31 aa, except B2_E[a], which has only 10 LRR. All members of S_H3-CNL family except A2_E[a] have an open read frame.

Table III. 1 Exon, intron size (bp) and protein size (aa) of the S_H3-CNL members identified in the three analyzed genomes.

Copy	genome	Exon 1	Intron	Exon 2	Protein (aa)
A_1	E[a]	1042	272	1787	943
A_1	C[a]	1042	255	1787	943
A_1	C[c]	1042	270	1898	980
A_2	E[a]	1042	270	1908	955
A_2	C[a]	1042	270	1820	954
A_2	C[c]	1042	269	1823	954
A_3	E[a]	1042	268	2003	1015
A_3	C[c]	1042	256	1865	969
B_1	E[a]	1042	258	1865	969
B_2	E[a]	1042	157	1703	915
B	C[a]	1042	260	1895	970
B	C[c]	1042	260	1874	972


100
Coiled-coil (CC)
EDVID motif
S_H3-CNL_A2_C^a MASEAVTLALGTVTDILAEEARFLFGVADQVKELEVELIGMQRLLKDADKKQLNDSTVRNYVRKIRRLAYRTEDVLEEFAVEIESRRRGHGFRKAFRRFA
S_H3-CNL_A2_E^a
S_H3-CNL_A2_C^c
S_H3-CNL_A3_E^a
S_H3-CNL_A1_C^a
S_H3-CNL_A3_C^c
S_H3-CNL_A1_E^a
S_H3-CNL_B1_E^a
S_H3-CNL_B_C^a
S_H3-CNL_B_C^c
S_H3-CNL_B2_E^a
S_H3-CNL_A1_C^c
NBS domain
200
S_H3-CNL_A2_C^a GLVSEGTALHRVGSEIASIIAGINSITKNLQTYGVIALSSTEDGQSSNARLDQNQQRLRQTYPHQVEEYFVGMEDDIRQLVSLITDERIRSHRVISVYGM
S_H3-CNL_A2_E^a
S_H3-CNL_A2_C^c
S_H3-CNL_A3_E^a
S_H3-CNL_A1_C^a
S_H3-CNL_A3_C^c
S_H3-CNL_A1_E^a
S_H3-CNL_B1_E^a
S_H3-CNL_B_C^a
S_H3-CNL_B_C^c
S_H3-CNL_B2_E^a
S_H3-CNL_A1_C^c
300
P-loop/kinase-1
RNBS-A
kinase-2
S_H3-CNL_A2_C^a GGLGKTTLARKIYKHIEVERAFKQFAWVSVTQQCNTMTVFRDLLKQLVPDERKESVEKMDERELVGELYKVQKETKSLVVLDDLWEIEDWKRLSVAFPFA
S_H3-CNL_A2_E^a
S_H3-CNL_A2_C^c
S_H3-CNL_A3_E^a
S_H3-CNL_A1_C^a
S_H3-CNL_A3_C^c
S_H3-CNL_A1_E^a
S_H3-CNL_B1_E^a
S_H3-CNL_B_C^a
S_H3-CNL_B_C^c
S_H3-CNL_B2_E^a
S_H3-CNL_A1_C^c
400
RNBS-B
RNBS-C
hydrophobic domain
S_H3-CNL_A2_C^a EADSKILITTRNQKLAEVEFPYPLNLLNEDEGWELLQKRAFAKRNGADCESGPRLEAVGRAIVRKCGNLPLAISAIGGVLSQKTSLEEWETVKNDVDSYI
S_H3-CNL_A2_E^a
S_H3-CNL_A2_C^c
S_H3-CNL_A3_E^a
S_H3-CNL_A1_C^a
S_H3-CNL_A3_C^c
S_H3-CNL_A1_E^a
S_H3-CNL_B1_E^a
S_H3-CNL_B_C^a
S_H3-CNL_B_C^c
S_H3-CNL_B2_E^a
S_H3-CNL_A1_C^c
500
RNBS-D
S_H3-CNL_A2_C^a RMSEGGKEEGYGAVLQVLALSYDELPYHLKPCFLYLGQYREDEDIDAEMLYRMWTAEGMVSSDHRRKGETLTDVAERYLYEMASRSMLQVKFYEFSTSRK
S_H3-CNL_A2_E^a
S_H3-CNL_A2_C^c
S_H3-CNL_A3_E^a
S_H3-CNL_A1_C^a
S_H3-CNL_A3_C^c
S_H3-CNL_A1_E^a
S_H3-CNL_B1_E^a
S_H3-CNL_B_C^a
S_H3-CNL_B_C^c
S_H3-CNL_B2_E^a
S_H3-CNL_A1_C^c
600
MDL motif
Spacer region
S_H3-CNL_A2_C^a VESCYLHDLMRDFCLA-GKEVEFLKLLDFRGGNDPLSDYSTERDDCTPRCSIHMEDGKKHCLGD-----VDSMISMALEASGQLRSLTLSGGTERRTASI
S_H3-CNL_A2_E^a
S_H3-CNL_A2_C^c
S_H3-CNL_A3_E^a
S_H3-CNL_A1_C^a
S_H3-CNL_A3_C^c
S_H3-CNL_A1_E^a
S_H3-CNL_B1_E^a
S_H3-CNL_B_C^a
S_H3-CNL_B_C^c
S_H3-CNL_B2_E^a
S_H3-CNL_A1_C^c

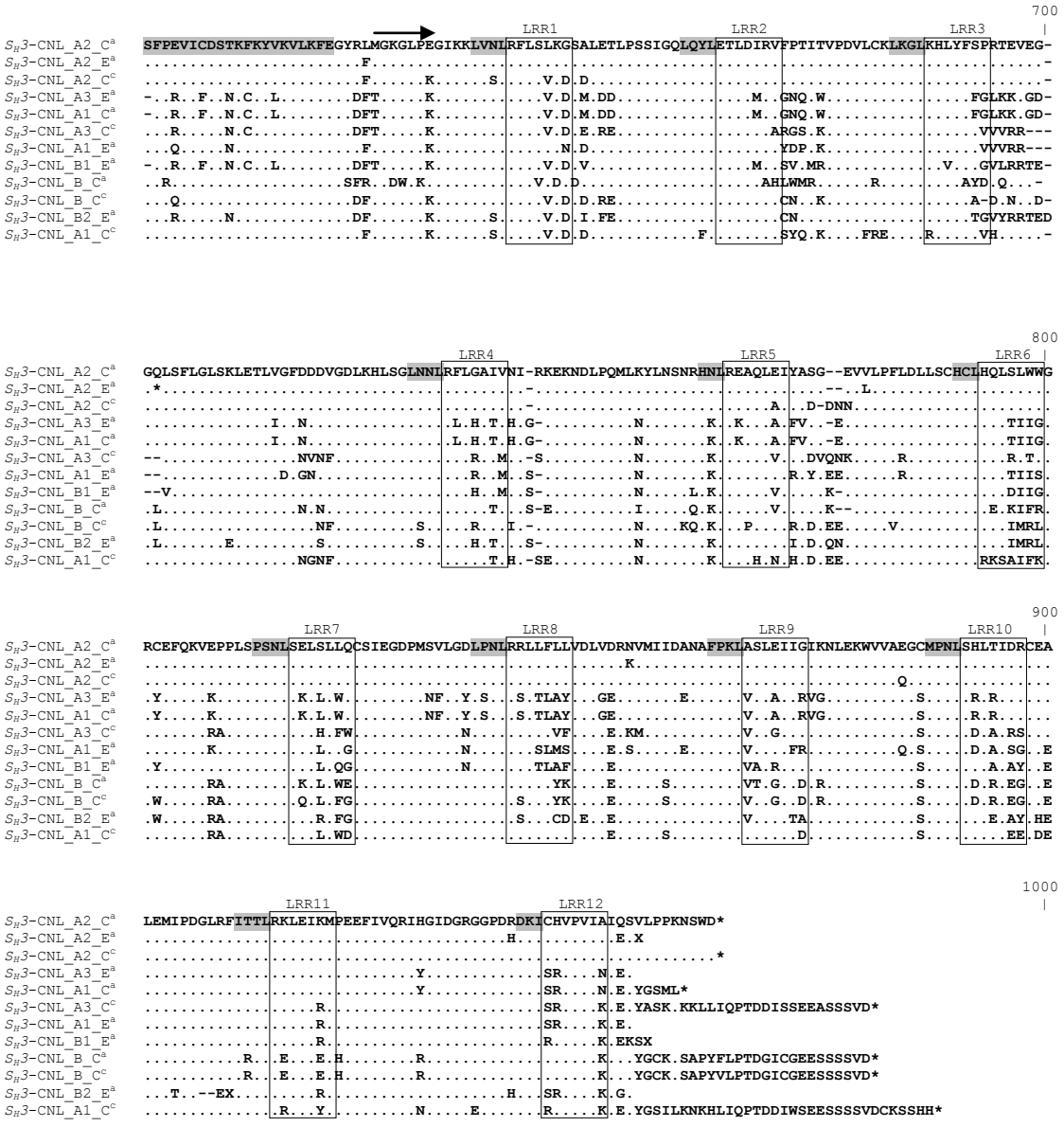


Figure III. 6 Alignment of the predicted amino acid sequences from S_H3-CNL members The coiled-coil domain is in rectangle. The motifs in NBS domain are underlined: P-loop/kinase 1, RNBS-A, kinase II, RNBS-B, RNBS-C, hydrophobic domain. The first sequence is shown in full, while other proteins only those amino acids that differ from the first one are indicated. The xxLxLxx motif in the LRR domain is boxed, where L is any aliphatic amino acid and x is any aminoacid. Gaps introduced at alignment are indicated by dashes, while asterisks indicate the presence of stop codons. NBS probe used in southern hybridization is highlighted in gray. The tryptophan residue (W), specific to the non-TIR–NBS–LRR class of plant disease R gene, located at the end of the kinase 2 motif (Meyers et al., 2004) is presented in bold face in a square.

Cloning of S_H3-CNL_A2 members from diploid species of coffee

To study interspecific diversity, the S_H3-CNL_A2 member was cloned from six coffee species (*C. anthonyi*, *C sp.* Congo, *C. canephora*, *C. eugenioides*, *C. liberica*, *C. pseudozanguebarie*). The cloned fragments were around 4 kb in size. Their sequences were determined and compared with those from C^a, E^a and C^c genomes.

Sequence diversity analysis of the S_H3-CNL family

Using the GENECONV program (Sawyer, 1989), significant traces of gene conversion were detected among the member of the S_H3-CNL family, both in *C. arabica* and *C. canephora* (Table III. 2). Among the eight different gene conversions detected, six occurred between the first exon and the intronic region. Moreover, two of the detected gene conversion events involved inter subgenomic exchanges. The DNA sp program (v.5) was used to estimate polymorphism among the four S_H3-CNL members in the genome of *C. canephora* species (C^c). The highest level of DNA polymorphism was detected in the LRR domain (π=0.17, 0.20 and 0.15) while the most conserved regions were in the NBS domain, especially in the P-loop, Kinase 2 and hydrophobic domains (Figure III.7).

Table III. 2 Gene conversions detected among S_H3-CNL members.

Sub-genome analyzed	S_H3-CNL members	BC KA pValue	Begin	End	Length (Nc)
C^c	A1_C^c x A2_C^c	0.00000	1269	2263	994
C^c	A1_C^c x B_C^c	0.00057	392	1217	825
C^a	A1_ C^a x A2_ C^a	0.00246	2974	3075	101
C^a	A1_E^a x A2_ E^a	0.00000	238	1176	938
E^a	A3_E^a x B2_E^a	0.00127	396	1317	921
E^a	A1_E^a x B1_E^a	0.00398	2041	2223	182
C^a + E^a	A1_C^a x B2_E^a	0.00042	550	1300	750
C^a + E^a	A3_E^a x B2_E^a	0.00049	396	1317	921
C^a + E^a	A3_E^a x A1_C^a	0.00103	550	1300	750

BC KA P (Bonferroni-corrected Karlin–Altschul (*p* values)) Begin, first nucleotide of the potential converted region; End, last nucleotide of the potential converted region; Length, length of the converted region (Nc).

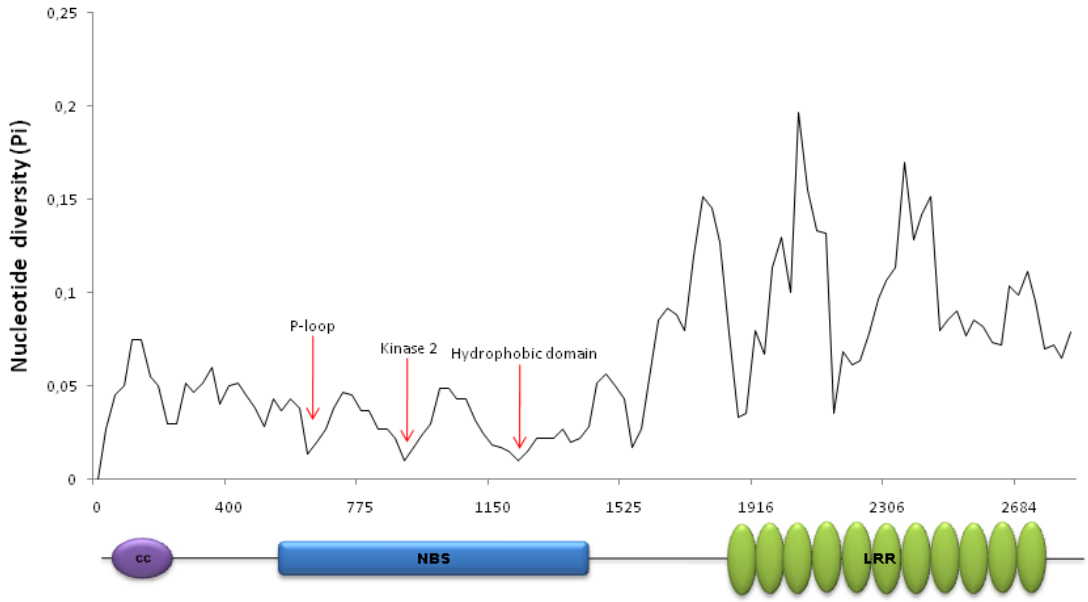


Figure III. 7 Nucleotide diversity among S_H3-CNL members from *C. canephora:* Nucleotide diversity (Pi) is the average number of nucleotide differences per site between two sequences calculated by DnaSP v.5. Nucleotide diversity was calculated using the sliding window method where a window (segment of DNA) is moved along the sequences step by step. The parameter is calculated in each window, and the value is assigned to the nucleotide at the midpoint of the window. Both the default values were used: window length of 100 sites, and step size of 25 sites (midpoint). The alignment gaps were not counted in the window length (or slide).

To check the type of selection that acted on genes in the S_H3-CNL family, the ratio between non-synonymous (Ka) and synonymous substitutions (Ks) was estimated using DNAsp v.5. The Ka/Ks substitution rate was calculated for each pair between ortholog and/or paralog members in *C. arabica* and *C. canephora* species. We also calculated the Ka/Ks between each pair of A2 members cloned from diploid coffee species together with A2 members from sequenced genomes (*C. arabica* and *C. canephora*). The analysis was performed on the complete coding sequence as well as on different domains (CC, NBS, LRR). Analysis also focused on codons encoding the solvent-exposed amino acids of the β-strand/β-turn motifs (x residues in xxLxLxx motifs) in the LRR domains.

Strong evidence for positive selection (Ka/Ks>1) was observed only for residues in xxLxLxx motifs. Among the 66 pair combinations between 12 BAC derived R-genes, 20 involving B members were under positive selection (Figure III.8). When the same region was analyzed among orthologous A2 members, no significant Ka/Ks>1 was found (Figure III.9) but 3 Ka/Ks < 1 were observed.

DISCUSSION

Organization and evolution of the S_H3-CNL family

Comparative analyses of R-gene clusters across different haplotypes or species demonstrated that the evolution of resistant genes is a dynamic process mostly involving duplication, deletion, sequence exchange, point mutation, diversified selection, recombination, gene conversion and retroelement insertion (Dai et al., 2010; David et al., 2009b; Innes et al., 2008; Kruijt, 2004; Kuang et al., 2008; Kuang et al., 2005b; Noel, 1999; Parniske and Jones, 1999; Seah et al., 2007b; Xiao, 2004). The cluster arrangement of R-genes represents an important reservoir of diversity and a source of genetic variation allowing the generation of novel resistance specificities via gene conversion, gene duplication, unequal crossing-over, ectopic recombination or diversifying selection (Hulbert et al., 2001; Michelmore and Meyers, 1998). To explore the organization and to characterize the mechanisms involved in the evolution of the S_H3 locus, where a putative R-gene cluster was identified (Cenci et al., 2010) a ~550 kb sequence was analyzed in three coffee genomes, E^a and C^a from *C. arabica* and C^c from *C. canephora*. Sequence analysis revealed the presence of a variable number of NBS-LRR genes belonging to the CC subclass at the S_H3 locus. All these genes belong to the same family (hereafter called S_H3-CNL family). Sequence analysis of regions flanking the S_H3-CNL genes helped determine the orthology relationship among the copies in different genomes. At the same time, several traces of ancient duplications made it possible to trace back the duplication/deletion events which, consistently with the birth and death evolution model, shaped the S_H3 locus from the most recent common ancestor of all S_H3-CNL copies. Since the structure of the S_H3 locus was well conserved in all three *Coffea* genomes analyzed, one can conclude that the origin of most of the S_H3-CNL copies predates the divergence between *Coffea* species.

Homologs of S_H3-CNL genes were found in several dicot species, but comparative genomics failed to find a CNL R-gene in the orthologous regions of three Rosid species (Cenci et al., 2010) and *Solanum lycopersicum* (order Solanales) which belongs as the *Coffea* species (order Rubiales) to the Asterid clade. Consequently, it can be concluded that the ancestral S_H3-CNL copy was inserted in the S_H3 locus after the divergence between Solanales and Rubiales lineages.

In a similar study by David et al. (David et al., 2009b) the authors compared the B4 locus of *Phaseolus vulgaris* (that has 26 CNL genes) with three sequenced legume genomes, *Medicago trunculata* (*Mt*), *Lotus japonicas* (*Lj*) and *Glycine max* (*Gm*). Their analysis revealed that conserved microsynteny existed among legumes species, except for the CNL sequences, which appeared to be completely absent in the corresponding regions of *Mt* and *Lj* and only a truncated CNL was found in

syntenic regions of *Gm* (David et al., 2009b). They suggested that CNL were inserted in the ancestor of the B4 locus after *Mt, Lj,* and *Pv* diverged but before the divergence of *Pv* and Gm through an ectopic recombination event between non-homologous chromosomes. Phylogenetic analysis including those legume CNL sequences and all known *Pv* B4-CNL sequences supported this hypothesis (David et al., 2009b).

Structural instability induced by repetitive mobile elements is one of the mechanisms that could lead to diversification into R gene families. The presence of very similar sequences increases the possibilities of mispairing during recombination, giving rise to unequal crossovers and interlocus gene conversions (Mcdowell and Simon, 2006; Meyers et al., 2003a; Michelmore and Meyers, 1998; Richter and Ronald, 2000). However, the edges of the duplications involved in the birth of new S_H3-CNL copies were not related to mobile elements and mobile elements identified in the region did not appear to play a role in the structural evolution of the S_H3 locus of *Coffea* species.

Gene conversion (i.e. the substitution of a portion of a gene sequence by the homologous sequence of another related gene) is more frequent among members of highly similar, tightly clustered families (Mondragon-Palomino and Gaut, 2005). Gene conversion is a common phenomenon and it has been detected between paralogs in many R-gene clusters (Caicedo, 1999; Cooley et al., 2000; Dodds et al., 2001; Ellis et al., 1999b; McDowell et al., 1998; Meyers, 1998; Noel, 1999; Parniske, 1997; Sun et al., 2001; Van der Hoorn et al., 2001). Sequence exchanges between different sub-genomes have previously been detected in a R1 resistance-gene cluster of one CNL subfamily in allohexaploid, *Solanum demissum* (Kuang et al., 2005a). In that study, seven of the 17 sequence exchanges among R1 homologs occurred between different genomes. Two explanations were proposed: first, sequence exchanges among different haplotypes could be generated through gene conversions or alternatively, they might have occurred through recombination before speciation and have been conserved in *S. demissum* (Kuang et al., 2005a). At the S_H3 locus, gene conversion events were detected between paralogs in all three coffee genomes analyzed and also between members of the two sub-genomes of *C. arabica.*

Conversion events were detected between S_H3-CNL members independently of their orientation (i.e. between members in region A and B).The inverted orientation of the loci might allow rare interlocus gene conversion or unequal exchange while minimizing the risk of gross chromosomal rearrangement (Parniske and Jones, 1999). The gene orientation in a cluster of NBS-LRR has also been studied in rice (Hulbert et al., 2001) and *A. thaliana* (Mondragon-Palomino and Gaut, 2005). These studies demonstrated that conversion can occur between genes in the same or in opposite orientation, however conversion is more frequent in gene families arranged as direct repeats because they have higher similarity than those found in opposite orientation.

In addition, gene conversion appears to be more frequent in perennial than in annual plants. Yang et al. (2008), compared the gene conversion events among NBS-encoding genes in two perennial and two annual plants. A total of 823 and 468 gene conversion events involving 299 and 187 NBS-encoding genes were detected in grapevine and poplar, respectively, while only 143 and 81 gene conversion events were detected in *Arabidopsis* and rice, respectively (Yang et al., 2008b). Since the long-generation time of woody species slows down the accumulation of evolutionary change, the authors suggested that an excess of recent duplications and a higher conversion rate in grapevine and poplar could generate novel resistance profiles to compensate for life history traits. According to Kuang et al. (Kuang et al., 2004; Kuang et al., 2005b) S_H3-CNL members should be classified as evolutionary type I (fast evolving genes) since several conversion events were detected between members.

Effect of selection on molecular evolution of the S_H3-CNL family

Natural selection influences the molecular evolution of sequences by increasing or reducing the fixation probability of a given mutation which, respectively, increases or reduces the fitness of the individuals carrying it. The effect of natural selection on a gene sequence can be investigated by analyzing nucleotide substitutions that occurred between two variants of this gene. Since synonymous substitutions (i.e. nucleotide substitutions that do not change the amino acid sequence) are supposed to not modify the phenotype, their accumulation is considered not to be influenced by natural selection. Conversely, non-synonymous substitutions (nucleotide substitutions that modify coded amino acids) could increase, reduce, or not influence the fitness of the individuals carrying it; consequently, their accumulation could be influenced by natural selection. The ratio of non-synonymous (Ka) to synonymous (Ks) substitution rates could be used to infer the effect of natural selection of a given gene or a part of it. When Ka and Ks have similar values ($Ka/Ks \approx 1$), one could infer a neutral effect of selection; when Ka is significantly lower than Ks ($0 < Ka/Ks < 1$), it could be deduced that the selection purges the gene sequence of most non-synonymous substitutions (purifying selection); finally, when Ka is significantly higher than Ks ($Ka/Ks > 1$), the selection is assumed to favor fixation of new variants (positive or diversifying selection) (Bergelson, 2001; Hurst, 2002).

In many NBS-LRR genes, analysis of corresponding proteins revealed high non-synonymous:synonymous substitution ratios in the leucine-rich (LRR) domain, mainly concentrated on the putative solvent-exposed residues, indicating that the LRR domain is subject to positive selection for amino acid diversification, (Botella, 1998; Dodds et al., 2001; Ellis et al., 2000a; Ellis et al., 1999a; Geffroy et al., 2009; McDowell et al., 1998; Meyers, 1998; Michelmore and Meyers, 1998; Parniske, 1997; Shen et al., 2002; Yahiaoui et al., 2006). These results are consistent with the

observation that nucleotide polymorphisms found in the leucine-rich (LRR) region of *R* genes are often responsible for pathogen specificity (Ellis et al., 2000a).

In the S_H3-CNL family, significant positive selection was only detected when the Ka/Ks analysis was focused on solvent-exposed residues (i.e. the x residues in xxLxLxx motif from LRR domain) most frequently among paralog members. Conversely, when larger regions were considered, the effect of natural selection was diluted and not detectable.

In the co-evolutionary arms race between hosts and their pathogens, genes involved in their interaction are expected to evolve under positive selection. The positive selection detected in the solvent-exposed residue of the S_H3-CNL members could indicate involvement in recognition of pathogen attack.

CONCLUSIONS

The S_H3-CNL family appears to have evolved following the birth-and-death model, since duplications and deletions were inferred in the evolution of the S_H3 locus. Gene conversion between paralog members from the same or different sub-genomes and positive selection appear to be the major forces influencing the evolution of S_H3-CNL in coffee trees.

MATERIALS AND METHODS

Plant material and DNA extraction

The cv. IAPAR 59 of *Coffea arabica* and six *Coffea* species were analyzed in this study: *C. canephora* (IF200), *C. anthonyi* (OD68), *C sp.* Congo (OB66), *C. eugenioides* (DA54), *C. liberica* (EA67), *C. pseudozanguebarie* (H66). Genomic DNA was isolated from leaves of greenhouse grown plants located at IRD (*Institut de Recherche pour le Développement*) Montpellier, France. Leaves were frozen in liquid nitrogen and DNA was extracted using a CTAB procedure (Doyle and Doyle, 1987) with modified extraction buffer (3% CTAB, 1.4 mM NACl, 100 mM Tris HCl, 20 mM EDTA, pH 0.8).

BAC sequences

Several Bacterial Artificial Chromosome (BAC) clones spanning the S_H3 locus were isolated from a *C. arabica* (IAPAR59) (Noir et al., 2004) and a *C. canephora* (HD-200-94) (unpublished data) libraries. Based on fingerprint data and overlapping sequence analysis, BAC sequences were assembled in contigs specific to the *C. arabica* and *C. canephora* genomes (hereafter called C^c for *C. canephora* genome; E^a and C^a for "eugenioides" and "canephora" sub-genomes of *C. arabica*) (Lashermes et al. 2010). Gene annotation of the BAC was already available (Cenci et al., 2010).

Primer design and cloning procedure

Orthologous specific primers to amplify A2 members of the S_H3-CNL family from wild *Coffea* species were designed based on sequence alignments of A2 members in *C. canephora* and *C. arabica*: A2_Left: 5'-CCTTGATAAGAAACATGAATGAAATACACGA-3' and A2_right 5'-AAGGATAAATGAGAAGAACTACTGAGCCTG-3'. DNA amplification was performed with Expand^{TM} 20Kb^{plus} PCR System (Roche Applied Science, Mannheim Germany). PCR were performed as follows: one cycle of 1 min at 95 °C, 10 cycles of 10 sec at 94 °C, 45 sec at 50 °C, 5 min at 68 °C followed by 20 cycles of 10 sec at 94 °C, 45 sec at 50 °C, 7 min at 68 °C plus 10 sec per cycle, and final extension of 7 min at 68 °C. A10 µl aliquot from each PCR amplification was analyzed by electrophoresis in a 1.2% agarose gel. The amplicons were gel-stained using Crystal violet and the DNA bands were purified using a S.N.A. P™ purification column (Invitrogen Carlsbad, CA). The PCR products were cloned into the pCR®-XL-TOPO® kit from Invitrogen and chemically competent cells (Invitrogen Carlsbad, CA) according to the manufacturer's protocol. Eight colonies were randomly selected for screening. Colony PCR of eight random samples was used to select clones containing the complete amplicon. For this purpose, two new primers were designed to amplify the extremities of the genes in combination with the primers used to amplify the whole gene: 5'-CGACAGTGGGAACGAAACCC-3'combined with A2_Left and 5'-TGGAGGACCGGATCATGAACA-3' combined with de A2_RIGHT. The colony PCR was performed as follows: 10 min at 94 °C, followed by 30 cycles of 30 sec at 94 °C, 30 sec at 55 °C, 4 min at 72 °C and final extension of 10 min at 72 °C. The colonies shown to contain the complete insert were transferred to 5 ml LB broth with 50 µg/ml kanamycin and incubated at 37 °C overnight. Plasmid DNA was isolated using Promega Wizard® *Plus* Minipreps DNA purification System (Promega Corporation, Madison, WI, USA) according to manufacturer's instructions. Two independent PCR and sequencing were carried out to ensure quality.

Sequencing and analysis of cloned S_H3-CNL members

Plasmid DNA was sequenced at Genome Express (Grenoble, France) using M13-universal-forward and reverse primers and five other internal primers were designed using the Primer3 program (Whitehead Institute, USA) to allow whole gene sequencing. INT1-L: 5-TCCATCGTCCAAGATACAGC-3, INT2-L: 5-TTTGTTGGGATGGAAGATGA-3, INT3-L: 5-GCTGGGAGTTGCTTCAAAAG-3, INT4-L: 5-TCGAATGTGGACAGCAGAAG-3, INT5-L: 5-GCCTTGGAGACACTTCCATC-3. *The cloned sequence contigs were assembled using the Staden package (Staden, 1996).* The complete sequences of each clone were aligned using Bioedit v.7.0 (Hall, 1999).

Southern blot analysis

Southern blot analysis was performed as follows: 20 µg of genomic DNA was extracted as described above and digested with a restriction enzyme (only *Eco*RI for the panel of diploid species *Eco*RI, *Dra*I and *Bam*HI for the *Coffea arabica* cv. IAPAR-59) and separated by agarose gel electrophoresis. The digested DNA was transferred to Hybond-N+ nylon membranes for Southern hybridization as described in Noir et al. (Noir et al., 2004). S_H3-CNL family specific probe was obtained by PCR amplification using primers designed on the NBS domain (left primer: 5'-CGGTCTCGGTAAGACCACTC-3'and right primer 5'-CCTCTGCAAATGGAAATGCT-3'). The amplified 516 bp fragment was labeled with [32P]-dATP according to the manufacturer's recommendations (Megaprime DNA Labelling Systems kit, Amersham) and used as probe in the hybridization experiment as described in Sambrook et al. (Sambrook et al., 1989).

Motif predictions

SMART protein motif analyses (http://smart.embl-heidelberg.de) and Pfam database (http://pfam.sanger.ac.uk/search/sequence) were used to detect motifs in the S_H3-CNL genes. A COIL with a threshold of 0.9 was used to specifically detect CC domains (Lupas et al., 1991).

Gene Conversion

In order to check the possibility of conversion events among the S_H3-CNL members, alignments of sequences from *C. arabica* and *C. canephora* species were analyzed with the GeneConv

program (Sawyer, 1989) using the default settings (in particular, mismatches within fragments were not allowed). The program tests for gene conversion by finding identical fragments between pairs of sequences in a nucleotide alignment. Pairwise P values are assigned based on the comparison of each fragment with the maximum fragment length that is expected from the sequence pair by chance (Sawyer, 1989). Pairwise P values were obtained by the method of Karlin and Altschul (Karlin and Altschul, 1993).

Sequence evolution

Protein sequences were manually aligned with the BioEdit program. The amino acid sequence alignments were used to guide the alignments of nucleotides using MEGA version 4.1 (Tamura et al., 2007). Nucleotide diversity (π) was calculated by DnaSP v.5 (Librado and Rozas, 2009) where each paralog was considered as an independent allele of population.

The *Ka/Ks* ratio was estimated by DnaSP based on Nei and Gojobori's equation (Nei, 1987) for full-length CDS for specific domains: (CC, NBS, LRR), for the xxLxLxx motifs in the LRR domain, and for the solvent-exposed residues (i.e. only the x residues in the xxLxLxx motif). P values were calculated and the significance level was compared at 0.05 and 0.01%.

Microsynteny Analysis

The search for putative gene orthologs of the genes at the S_H3 locus was performed by TBLASTN analysis on the tomato genome sequence (*Solanum lycopersicum*) available in Solanaceae Genome Network **(**www.sgn.cornell.edu*)*.

P Ka/ka	A1_E[a]	A1_C[a]	A1_C[c]	A2_E[a]	A2_C[a]	A2_C[c]	A3_E[a]	A3_C[c]	B1_E[a]	B_C[a]	B_C[c]	B2_E[a]
A1_E[a]		0.0577	0.0982	0.0838	0.0838	0.0405	0.0577	0.0606	0.0696	0.0097	0.0275	0.0383
A1_C[a]	3.09		0.0448	0.2066	0.2066	0.1503	N/A	0.1033	0.0139	0.0157	0.0080	0.0362
A1_C[c]	1.09	3.02*		0.2430	0.2430	0.1963	0.0448	0.0517	0.1212	0.1800	0.0987	0.0613
A2_E[a]	2.65	1.89	1.86		N/A	0.6055	0.2066	0.2294	0.0988	0.0950	0.2670	0.0503
A2_C[a]	2.65*	1.89	1.86	N/A		0.6055	0.2066	0.2294	0.0988	0.0950	0.2670	0.0503
A2_C[c]	3.52*	2.16	2.03	0.53	0.53		0.1503	0.1392	0.0563	0.0530	0.1487	0.0214
A3_E[a]	3.09	N/A	3.02*	1.89	1.89	2.16		0.1033	0.0139	0.0157	0.0080	0.0362
A3_C[c]	1.30	2.46	0.73	1.95	1.95	2.46	2.46		0.3088	0.0610	0.1039	0.1541
B1_E[a]	3.82	6.95*	2.25	2.49	2.49	3.14	6.95*	1.80		0.0203	0.0156	0.0475
B_C[a]	5.75**	5.28*	1.38	2.57	2.57	3.30	5.28*	3.19	4.55*		N/A	0.0076
B_C[c]	4.38*	7.16**	2.45	1.82	1.82	2.31	7.16**	2.75	5.66*	N/A		0.0652
B2_E[a]	4.03*	3.65*	0.78	3.45	3.45*	5.26*	3.65*	2.38	4.09*	7.49**	4.55	

Figure III. 8 Ka/Ks ratio in the solvent-exposed residue of S_H3-CNL members: The Ka/Ks ratio was calculated in the solvent-exposed residue of the LRR domain by pairwise comparison of S_H3-CNL members. Values below the diagonal are the Ka/Ks ratio and values above are the probability, significance level for Ka>Ks indicated by * P<0.05, ** P<0.01. N/A=not applicable.

P Ka/Ks	*C. arabica*_A2_C[a]	*C. arabica*_A2_E[a]	*C. canephora*_A2_C[c]	*C. canephora*	*C. anthonyi*	*C. congo*	*C. eugenioides*	*C. pseudozanguebarie*	*C. liberica*
*C. arabica*_A2_C[a]		N/A	0.6055	0.6055	0.6059	0.6059	0.963	0.874	0.577
*C. arabica*_A2_E[a]	0.00		0.6055	0.6055	0.6059	0.6059	0.963	0.874	0.577
*C. canephora*_A2_C[c]	0.53	0.53		N/A	0.4226	0.4226	0,.822	0.654	0.747
C. canephora	0.53	0.53	0.00		0.4226	0.4226	0.822	0.654	0.747
C. anthonyi	1.32	1.32	1.60	1.60		N/A	0.601	0.462	0.032
C. congo	1.32	1.32	1.60	1.60	0.00		0.601	0.462	0.032
C. eugenioides	0.98	0.98	1.13	1.13	0.53	0.53		0.937	0.006
C. pseudozanguebarie	1.09	1.09	1.30	1.30	2.18	2.18	1.07		0.291
C. liberica	0.77	0.77	0.85	0.85	0.17*	0.17*	0.12**	0.51	

Figure III. 9 Ka/Ks ratio in the solvent-exposed residue of S_H3-CNL_A2: The Ka/Ks ratio was calculated in the solvent-exposed residue of LRR domain by pairwise comparison of members of orthologous S_H3-CNL_A2. Values below the diagonal are the Ka/Ks ratio and values above are the probability, significance level for Ka>Ks indicated by * P<0.05, ** P<0.01. N/A=not applicable.

CHAPTER IV

Agrobacterium-MEDIATED GENETIC TRANSFORMATION OF *Coffea arabica* (L.) IS HIGHLY ENHANCED BY USING LONG-TERM MAINTAINED EMBRYOGENIC CALLUS

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Agrobacterium-mediated transformation of *Coffea arabica* (L.) is highly enhanced by using long-term maintained embryogenic callus

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ABSTRACT

Background: One of the challenges following the genome sequencing of several important crop plants is to determine the function of all the predicted genes. The main difficulty when applying gene validation approaches to woody species is the low recovery of transgenic plants from elite or commercial cultivars. Embryogenic calli have been frequently used as the target tissue for transformation, but the difficulty in producing or maintaining embryogenic tissues is one of the major problems encountered in genetic transformation of many woody plants, including *Coffea arabica*.

Results: We established the conditions for long-term proliferation of embryogenic cultures in C. arabica and a highly efficient and reliable *Agrobacterium tumefaciens*-mediated transformation method based on their utilization. The transformation protocol using LBA1119 harbouring pBin35SGFP was established by evaluating the effect of different parameters on transformation efficiency through GFP detection. Using embryogenic callus cultures, co-cultivation with LBA1119 OD_{600} = 0.6 for 5 days at 20°C enabled reproducible transformation. However, the maintenance conditions for the embryogenic callus cultures - particularly a high auxin:cytokinin ratio and the culture age (opt. for 7-10 month proliferation) - and use of the yellow callus phenotype were revealed to be the most important factors for achieving highly efficient transformation (> 90%). Transformation success was related to the abundance of proembryogenic masses (PEMs). All the regenerated plants were proved to be transformed by PCR and Southern blot hybridization.

Conclusion: Most of the progress achieved in increasing transformation efficiency was brought about by optimizing the production conditions of the explant much more significantly than with conventional optimization of co-cultivation conditions. The establishment of maintained embryogenic cultures and the optimization of their physiological status could be a valuable way of establishing reliable transformation conditions in other recalcitrant woody species.

BACKGROUND

Recent advances in plant genomics have greatly increased knowledge of gene structure, organization, evolution and biological functioning. The genome sequencing of several important crop plants such as wheat, sugarcane, tomato, potato, banana, eucalyptus and coffee, has already been completed or is in progress (NBCI Entrez Genome Project data base; http://www.ncbi.nlm.nih.gov/genomeprj). So far, the information generated will offer some tremendous new challenges in plant biology, including determining the function of predicted genes. For example, with the 120Mb genome of the model plant *Arabidopsis thaliana*, only 60% of the 33,518 unique genes initially predicted could have an experimentally assigned function (TAIR data base; http://www.arabidopsis.org/portals/genAnnotation/genome_snapshot.jsp). The most reliable way to assess gene function is engineering transgenic plants to generate gain-of-function or loss-of-function mutants (Curtis and Grossniklaus, 2003). Gene function analysis using plant transformation is well established in model plants. However, for many important crop plants, including most woody species, genetic transformation still remains a laborious and low-efficiency process.

The main difficulty when applying gene validation approaches to woody species is the low recovery of transgenic plants from elite or commercial cultivars (Gomez-Lim and Litz, 2004; Pérez-Clemente et al., 2004; Petri and Burgos, 2005). Apart from poplar - a model plant for woody plant transformation with overall transformation efficiencies between 15 and 36% (Cseke et al., 2007; Yevtushenko and Misra, 2010) - a low transformation efficiency has been reported in many woody species ranging from 1.5% to 4.7% in apple (Yao et al., 1995; Bolar et al., 1999; Seong and Song, 2007), 3.8 to 8.6% in apricot (Lopez-Noguera et al., 2009), around 3.5% in peach (Pérez-Clemente et al., 2004), 2% in leucaena (Jube and Borthakur, 2009) and less than 1% in cacao (Maximova et al., 2003) and coffee (Etienne et al., 2008).

It has frequently been reported that the choice of explants capable of transformation and regeneration is a crucial factor (Giri et al., 2004). Somatic embryogenesis is definitely an advantageous support for genetic transformation, because it potentially provides strong capacity for the regeneration of transgenic material and appears to be the most promising approach for introducing new genes into woody trees species. Embryogenic calli have been used as the target tissue for transformation in many woody plants, such as pine (Leeve et al., 1999; Tereso et al., 2006; Trontin et al., 2007), grapevine (Dhekney et al., 2008), rubber tree (Montoro et al., 2000), mango (Krishna and Singh, 2007), avocado (Cruz-Hernandez et al., 1998), American chestnut (Andrade et al., 2009) and coffee (Hatanaka et al., 1999; Leroy et al., 2000; Ribas et al., 2006a Albuquerque et al., 2009). Nevertheless, the production of embryogenic tissues remains a difficult step in woody species as it is a low-efficiency process, time consuming, with low reproducibility often greatly genotype-dependent.

Moreover, the conditions for long-term proliferation of embryogenic tissues that enable their maintenance and amplification have only been reported for very few species.

The decline or loss of embryogenic potential in plant tissue cultures is one of the major problems encountered in genetic manipulation, making the production of fresh callus for each transformation round time-consuming (Zhang et al., 2000; Sharma et al., 2005; Pniewski et al., 2006). Maintaining embryogenic tissues over a long period can avoid the need for frequent *in vitro* introductions that require a new, laborious and risky process for the selection of embryogenic tissues. Thus, by determining the best conditions for maintaining the regenerative potential of embryogenic tissues is extremely important with a view to using them in genetic transformation experiments. The establishment and maintenance of embryogenic cultures depend on the subculture interval, medium composition and tree species (Jain, 2006). The conditions for long-term proliferation of embryogenic callus have been achieved in some species such as the rubber tree (Montoro et al., 1994), Eucalyptus (Muralidharan and Mascarenhas, 1995), grape (Motoike et al., 2001), garlic (Hasegawa, 2002) and turfgrass (Liu et al., 2009). In rubber trees, long-term embryogenic cultures have made it possible to establish optimized genetic transformation conditions to study the function of genes involved in latex production (Blanc et al., 2006; Montoro et al., 2008).

In coffee, genetic transformation has been achieved using several explants (leaves, embryogenic callus, somatic embryos, protoplast, cell suspensions) and different approaches including *A. tumefaciens*-mediated transformation (Hatanaka et al., 1999; Leroy et al., 2000; Ogita et al., 2004; Ribas et al., 2006a), *A. rhizogenes*-mediated transformation (Alpizar et al., 2006; Kumar et al., 2006b), biolistic gene delivery (Boxtel et al., 1995; Ribas et al., 2005) and electroporation-mediated gene delivery (Fernandez and Medendez, 2003). The recovery of transgenic plants appears easier for the *Coffea canephora* species than for *C. arabica* (reviewed by (Kumar et al., 2006a; Ribas et al., 2006b; Etienne et al., 2008). The induction of embryogenic tissues in *C. arabica* is also longer and more difficult than in *C. canephora,* for which only protocols for their maintenance have been established.

Transgenic plants of *C. arabica* were successfully produced using embryogenic calli (Ogita et al., 2004; Ribas et al., 2006a; Albuquerque et al., 2009;) and nowadays this explant tends to be the one most used. However the protocols available so far, mainly using *A. tumefaciens,* are not reproducible and have revealed weak transformation efficiency, which greatly limits their potential use for routine gene function validation. The first example of the introduction of genes of agronomic interest in Arabica coffee was conducted by (Leroy et al., 2000). They regenerated transgenic coffee plants carrying the *CRY1-AC* gene from *Bacillus thuringiensis* which is effective against coffee leaf miner. The only work on functional validation by loss of function in Arabica coffee was developed by Ogita et al. (2004) who obtained transgenic coffee plants with suppressed caffeine synthesis using RNA interference (RNAi) technology through inhibition of a theobromine synthase gene (*CaMXMT1*).

The objective of our work was to demonstrate that reliable genetic transformation for functional genomics is achievable in a recalcitrant tree species such as *C. arabica* by establishing and monitoring long-term embryogenic cultures. An efficient *Agrobacterium*-mediated transformation protocol was set up by establishing co-cultivation conditions, the hormonal and salt balance of the embryogenic callus proliferation medium, and by determining the optimum morphological and histological characteristics and age of embryogenic calli used in transformation experiments. Finally, the presence and integration of transgenes was verified by PCR and southern blot analysis in the transgenic plants derived from transformed embryogenic cultures.

RESULTS

Explant and co-cultivation conditions

Different types of coffee tissues were tested under our transformation conditions; GFP detection was performed 30 days after co-cultivation to attest stable transformation events (Table IV.1). In this study, the detection of GFP activity was successfully applied to monitor the transformation efficiency for all the studied parameters. No GFP activity was detected in zygotic embryo fragments and a very low level was observed when using embryogenic cell suspensions. On the other hand, maintained friable embryogenic callus cultures were revealed to be an excellent support for genetic transformation, since significantly higher transformation efficiency (17%) was obtained with 4-month-old proliferating embryogenic calli using the same co-cultivation conditions. Using epifluorescence analysis, strong GFP activity was detected in embryogenic callus cultures 5 and 30 days after co-cultivation and in derived somatic embryos at all ontogenetic stages (Fig. IV.1).

Some other co-cultivation parameters that were demonstrated to be decisive in establishing an efficient *A. rhizogenes*-mediated transformation protocol (Alpizar et al., 2006) were tested to set up co-cultivation conditions with *A. tumefaciens* using specifically embryogenic callus cultures. Two concentrations of bacteria suspension were first compared for their efficiency in enhancing genetic transformation with embryogenic callus cultures (Table IV. 1). Using an undiluted *A. tumefaciens* suspension exhibiting an OD_{600} between 0.6-0.7 proved to be slightly better than the diluted bacterial solution (1/10). Similarly, we observed a weak increase in transformation efficiency by reducing the co-cultivation temperature from 27°C to 20°C (Table IV.1). However, the lower temperature during this period prevented bacterial overgrowth which made the decontamination process difficult and could lead to callus necrosis.

Table IV. 1 Effect of different co-cultivation factors on transformation efficiency

Co-cultivation factors	Treatments	Nb of co-cultivated explants	Nb transformed explants	Transformation efficiency (%)
Explant type	Zygotic embryo	88	0	0 ± 0
	Cell suspension (4 months)	192	4	0.02 ± 0.03
	Embryogenic callus culture (4 months)	240	41	17 ± 0.02
Agrobacterium conc. (OD_{600})	1 (OD_{600} = 0.6)	80	10	12.5 ± 11.1
	1/10	102	2	1.9 ± 4.1
Co-cultivation temperature (°C)	20	215	10	4.7 ± 4.3
	27	350	2	0.6 ± 1.2

*The transformation efficiency was analyzed by the proportion (p) of transformed calli (p=x/n), where x was the number of transformed calli and n the number of experiments. A 3 δ confidence limit for binomial distribution was calculated using the formula $p\pm3\sqrt{p(1-p/n)}$ level of confidence 99%

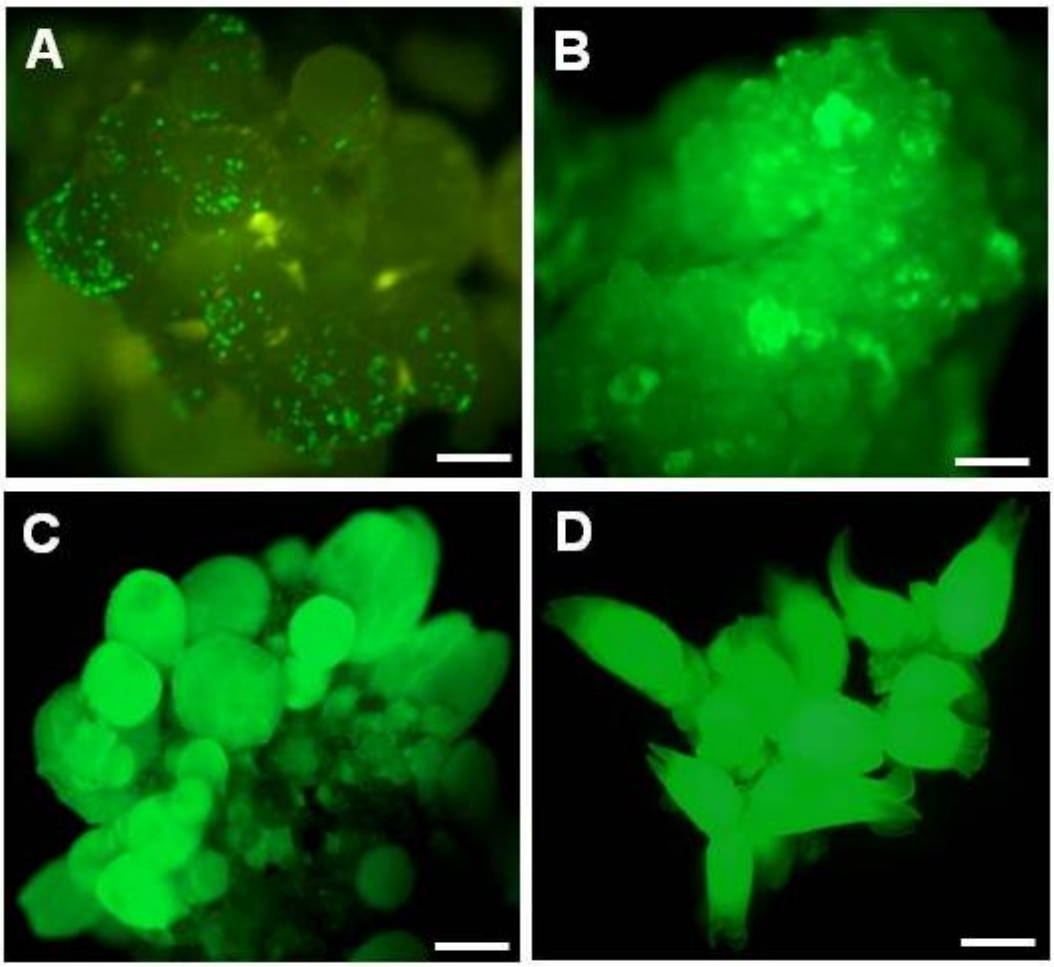

Figure IV. 1 GFP fluorescence in embryogenic callus cultures transformed with *A. tumefaciens* and in derived somatic embryos. Transient expression in embryogenic callus 5 days after co-cultivation, bar scale = 50 µM (A). Stable expression in embryogenic callus culture 30 days after co-cultivation, bar scale = 50 µM (B). Stable expression in globular and heart stage 6 months after co-cultivation, bar scale = 100 µM (C). Stable expression in torpedo mature somatic embryos 7 months after co-cultivation, bar scale = 500 µM (D).

Effect of the embryogenic culture proliferation medium composition on subsequent transformation

The composition of the nutritive medium used for embryogenic callus proliferation prior to genetic transformation can directly influence embryogenic callus physiological status, hence the success of transformation. We therefore tested different salt concentrations and auxin:cytokinin ratios for their effect on callus growth intensity - on which the availability of tissues suitable for transformation depends - and transformation efficiency. Successful transformation was only obtained with the diluted MS/2 and MS/4 salt concentrations (Table IV. 2). This later gave significantly higher transformation efficiency, but embryogenic callus growth was greatly affected by this much diluted salt concentration, as callus weight increase was 2 to 2.5 times less compared to the values obtained with other salt concentrations. On the other hand, MS and 1.5 MS media rich in salts were not appropriate for coffee transformation. The MS/2 salt concentration, which enabled efficient embryogenic callus proliferation and subsequent efficient transformation, was the condition used for the other transformation experiments. As regards the auxin:cytokinin ratio in the proliferation medium, we first observed that 6-BA had a strong negative effect on genetic transformation, as very efficient transformation was obtained without exogenous cytokinin (Fig. IV.2 A). It can be noted that 6-BA was not necessary either to sustain embryogenic callus proliferation, as acceptable growth intensity over 3 sub-cultures was obtained without 6-BA. Similarly to cytokinin, the 2,4-D concentration had a weak effect on embryogenic callus growth (IV.2 B). A relatively high auxin concentration (9 µM 2,4-D) in the embryogenic callus proliferation medium led to the highest transformation efficiency, confirming the positive effect of an auxinic environment on the pre-culture medium prior to coffee transformation.

Table IV. 2 Effect of different salts concentrations in the embryogenic callus culture proliferation medium on callus growth and transformation efficiency.

Salts concentration in the ECP proliferation media	Callus growth during a 4 week sub-culture (mg)	Nb of co-cultivated calli	Transformation efficiency (%)
MS/4	103 ± 20.7 b*	87	15.6 ± 6.6**
MS/2	267 ± 24.1 a	160	10.5 ± 14.1
MS	254 ± 60.5 a	100	0 ± 0
1.5MS	217 ± 53.6 a	160	0 ± 0

* Callus growth was measured by the difference between the final and initial weight of embryogenic cultures after a 4-week proliferation cycle. The initial weight was calibrated at 120 ± 10 mg. Each data corresponds to a mean ± SD from 4 measurements. We performed an ANOVA followed by the Tukey HSD test to determine the significant differences between the means of all treatments. Values with different letters are significantly different at $P \leq 0.05$. ** The transformation efficiency was analyzed by the proportion (p) of transformed calli ($p=x/n$), where x was the number of transformed calli and n the number of experiments. A 3 δ confidence limit for binomial distribution was calculated using the formula $p \pm 3\sqrt{p(1-p/n)}$ level of confidence 99%. Each value is the mean from 4 replicates.

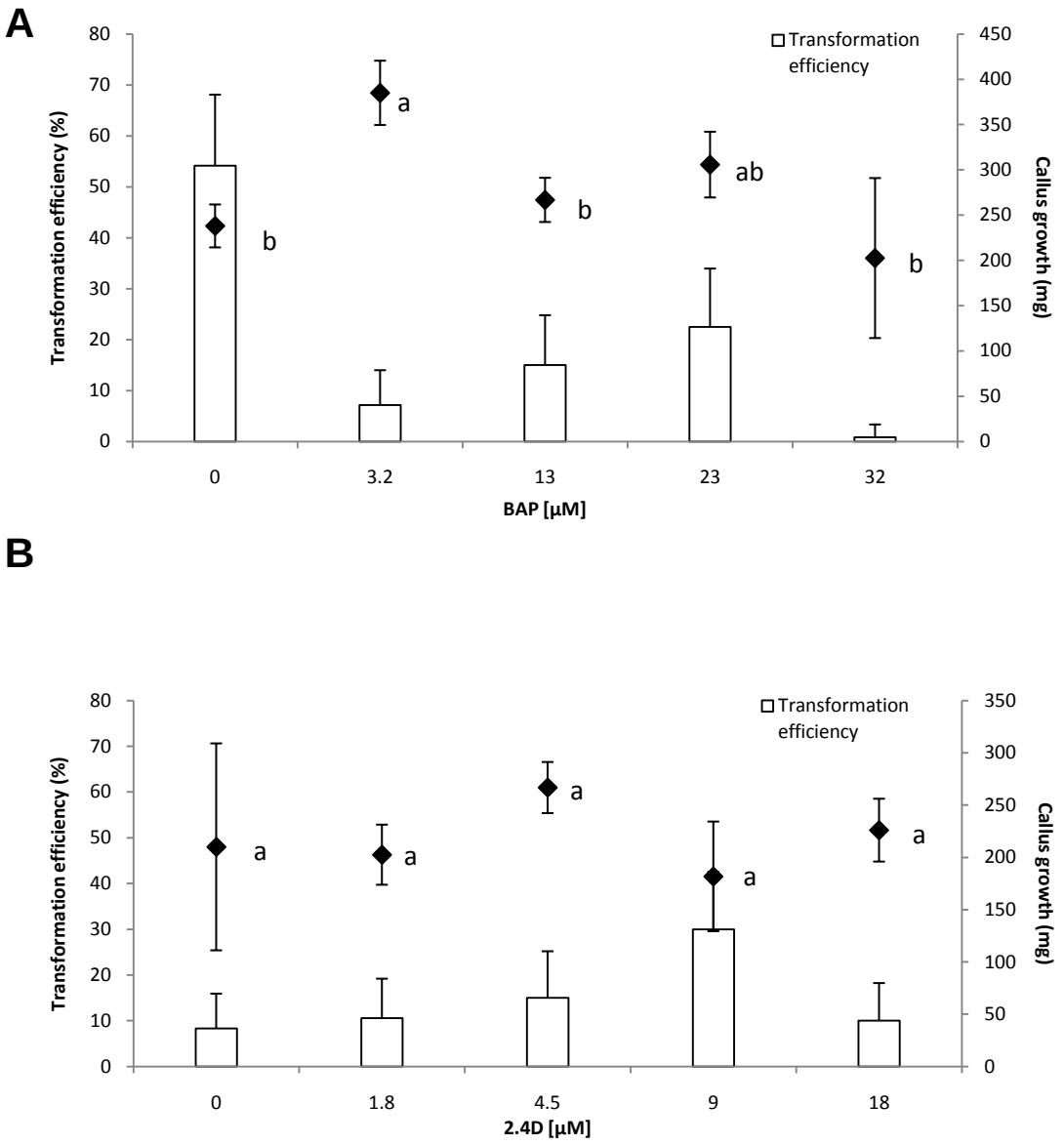

Figure IV. 2 Effect of 6-BA and 2,4-D concentrations in the proliferation medium on growth intensity and transformation efficiency of coffee embryogenic callus cultures. All the 6-BA concentrations were tested in the presence of 4.52 µM 2,4-D and all the 2,4-D concentrations were tested in the presence of 12 µM 6-BA. Callus growth was measured by the difference between the final and initial weight of embryogenic cultures after a 4-week proliferation cycle. The initial weight was calibrated at 120 ± 10 mg. Each data corresponds to a mean ± SD from 4 measurements. We performed an ANOVA followed by the Tukey HSD test to determine the significant differences between the means of all treatments. Values with different letters are significantly different at $P \leq 0.05$. Transformation efficiency was assessed by observing GFP epifluorescence 6 weeks after the end of co-cultivation. The transformation efficiency was analyzed by the proportion (p) of transformed calli ($p=x/n$), where x was the number of transformed calli and n the number of experiments. A 3 δ confidence limit for binomial distribution was calculated using the formula $p \pm 3\sqrt{p(1-p/n)}$ level of confidence 99%. All the transformation experiments were conducted independently in four replicates containing each 60 calli (240 calli/ hormonal combination).

In spite of the overall success of coffee transformation, we noticed the persistence of strong disparity for the same optimized conditions. Whereas for some jars almost all the callus produced hygromycin-resistant calli, in others no resistant callus was observed. We suspected that the variability observed in the transformation results came from the existence of an additional effect related to heterogeneity within the embryogenic material. Careful observation of the embryogenic callus cultures led us to distinguish between three callus phenotypes for the same culture conditions. All the phenotypes were friable but some differences in colour and texture could be seen. These phenotypes, called whitish, yellow and grey, are shown in Figs. IV. 3A, B, C. The grey one is more compact when compared to the yellow and the whitish corresponded to the most airy and fast-growing one. The whitish phenotype was the most frequent (60-70%) whereas the yellow and grey ones represented 25-35% and 5-10% of the population, respectively. Based on this observation, we started to separate the calli into the three batches according to the phenotype and tested them for genetic transformation. Figure IV.4 shows that no transformation event was observed using the grey callus phenotype and less than 1% for the whitish one. On the other hand, very high transformation efficiency (75%) was reached using the yellow friable calli, which was thus highly suitable material for coffee genetic transformation. Histological studies revealed important differences between the three embryogenic callus phenotypes (Fig. IV.3). The whitish phenotype (Fig. IV. 3 A) displayed a much loosened structure comprising isolated embryogenic cells, along with small sized pro-embryos (always <10 cells) [Fig. IV.3 D]. A proembryo segmentation and degeneration process was observed, corresponding to the initiation and rapid degeneration of somatic embryogenesis events. Cells constituting this type of callus did not exhibit a dense cytoplasm (not those belonging to proembryos) but had small starch grains and the visible mitosis stages suggested rapid divisions and growth (Fig. IV. 3 G). The yellow callus (Fig. IV. 3B) corresponded to a highly homogeneous tissue mainly comprising small cell aggregates similar to proembryogenic masses (PEMs) (Fig. IV. 3E). The cells exhibited a high nucleus:cytoplasm ratio and exhibited a voluminous and central nucleus, numerous small starch grains around the nucleus and a very dense cytoplasm rich in soluble and reserve proteins (Fig. IV.3 H). The cell walls were variable in thickness as is typically observed for embryogenic cells (Schwendiman et al., 1990; Verdeil et al., 2001). The grey phenotype (Fig. IV.3 C) was much more heterogeneous and constituted of a mix of degenerating tissues along with active and intermediate areas (Fig. IV.3 F). The cells were more vacuolated with a non-central nucleus, a small nucleolus and less abundant but bigger starch grains (Fig. IV.3 I).

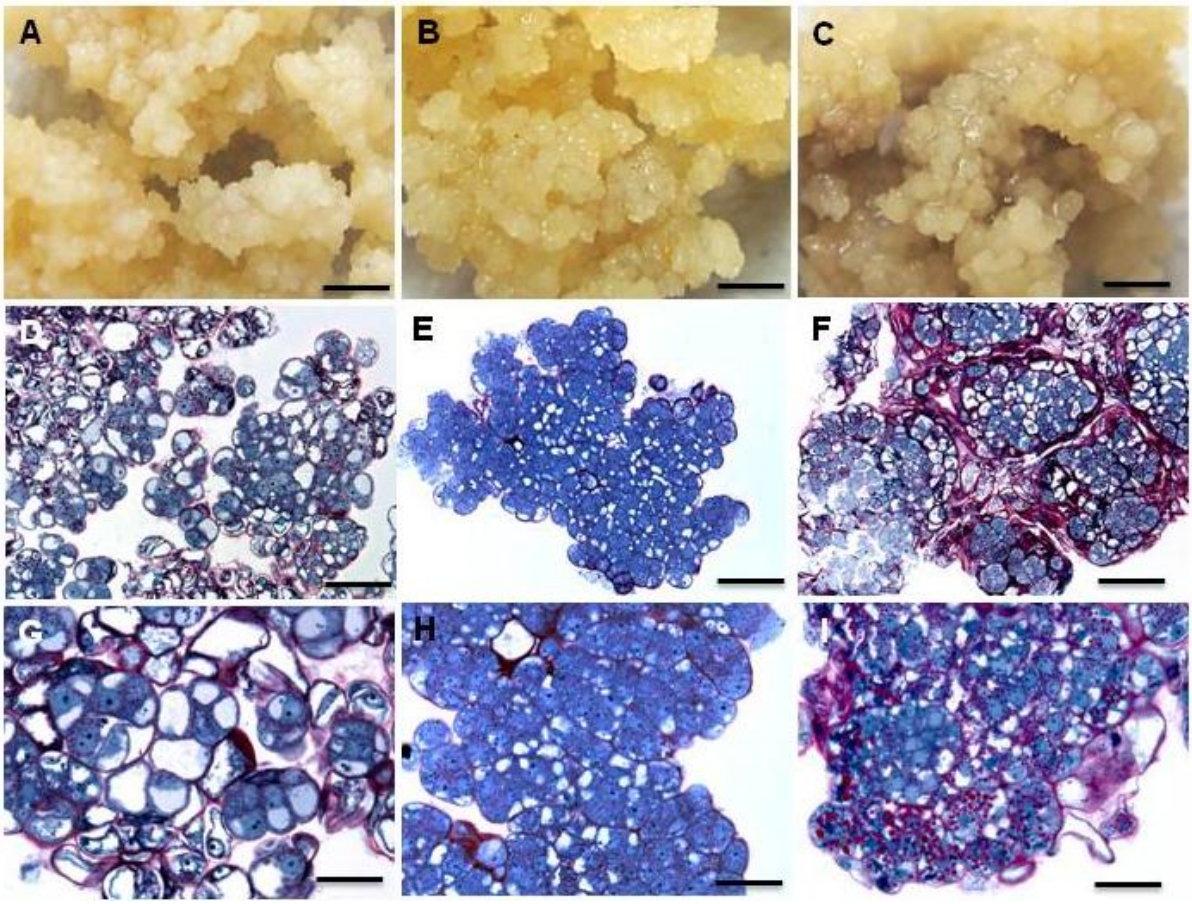

Figure IV. 3 Morphological and histological aspect of the different callus phenotypes observed in maintained embryogenic cultures assayed for coffee genetic transformation. Whitish type embryogenic callus: morphology, scale bar = 2 mm (A) and histological aspect (D), (G). Note the very loosened structure of the callus and the presence of proembryos (pe), isolated embryogenic cells (ec) along with abundant degenerating cells and proembryos (arrow), scale bar = 73 µM (D); observe the segmentation and degenerating process of proembryos (arrow) and the weakly dense cytoplasm of all cells, scale bar = 36.5 µM (G). Yellow type embryogenic callus: morphology, scale bar = 2 mm (B) and histological aspect (E); embryogenic callus comprising small cell aggregates similar to proembryogenic masses (PEMs); observe the strong homogeneity of the tissues, scale bar = 73 µM (E); note the high nucleus:cytoplasm ratio of all cells, the voluminous and central nucleus (n) and the numerous small starch grains (s) around the nucleus; observe in all cells the very dense cytoplasm rich in soluble and reserve proteins (blue staining), scale bar = 36.5 µM (H). Grey type embryogenic callus: morphology, scale bar = 2 mm (C) and histological aspect (F), (I); note the heterogenous callus structure comprising a mix of degenerating tissues (on the left) along with active (on the right) and intermediate areas, scale bar = 73 µM (F); most of the cells are vacuolated with a non-central nucleus (n), a small nucleolus (nl) and less abundant but bigger starch grains (s), scale bar = 30 µM (I).

All these observations are generally associated with degenerating tissues. Sub-culturing the different types of calli separately showed that each phenotype was maintained over the sub-cultures (data not shown). The stability of these embryogenic callus phenotypes offered an opportunity to select the transformation-competent yellow phenotype and easily eliminate the undesirable ones

during the proliferation process aimed at constituting stocks of highly competent embryogenic material with a view to genetic transformation experiments.

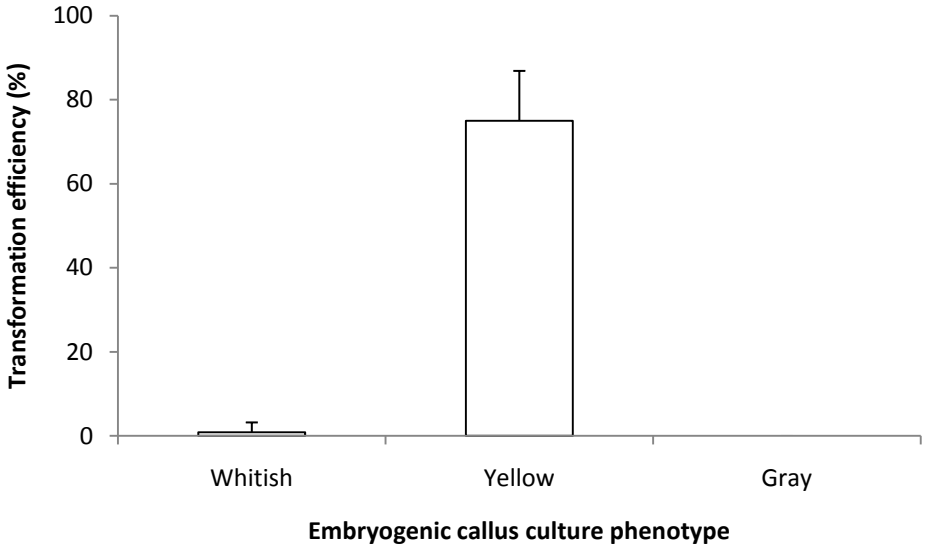

Figure IV. 4 Transformation efficiency depending on the phenotype of maintained embryogenic callus cultures. Transformation efficiency was assessed by observing GFP epifluorescence 6 weeks after the end of co-cultivation. The transformation efficiency was analyzed by the proportion (p) of transformed calli (p=x/n), where x was the number of transformed calli and n the number of experiments. A 3 δ confidence limit for binomial distribution was calculated using the formula $p \pm 3\sqrt{p(1-p/n)}$ level of confidence 99%. For each callus phenotype, all the transformation experiments were conducted independently in three replicates containing 40 calli (120 calli/phenotype).

Effect of culture age on transformation efficiency

Long-term maintenance of competent yellow type embryogenic callus was achieved by sub-culturing it every month on fresh gelled proliferation media. Embryogenic callus cultures with very variable proliferation durations (i.e. age of the culture) were transformed with *A. tumefaciens*. Figure IV.5 shows how the age of the embryogenic callus culture strongly affected transformation efficiency. Surprisingly, the best transformation efficiency (almost 100%) was achieved by using embryogenic callus cultures with at least 7 months of proliferation, and much lower transformation efficiency (around 15%) was obtained with the primary embryogenic callus. Transformation efficiency gradually

increased in line with the age of the embryogenic culture, with a maximum for 7 and 9 months maintained at a high level over a long period of time, since 70% efficiency was still obtained with 16-month-old embryogenic cultures. Twenty-six-month-old embryogenic cultures had almost completely lost their ability for genetic transformation.

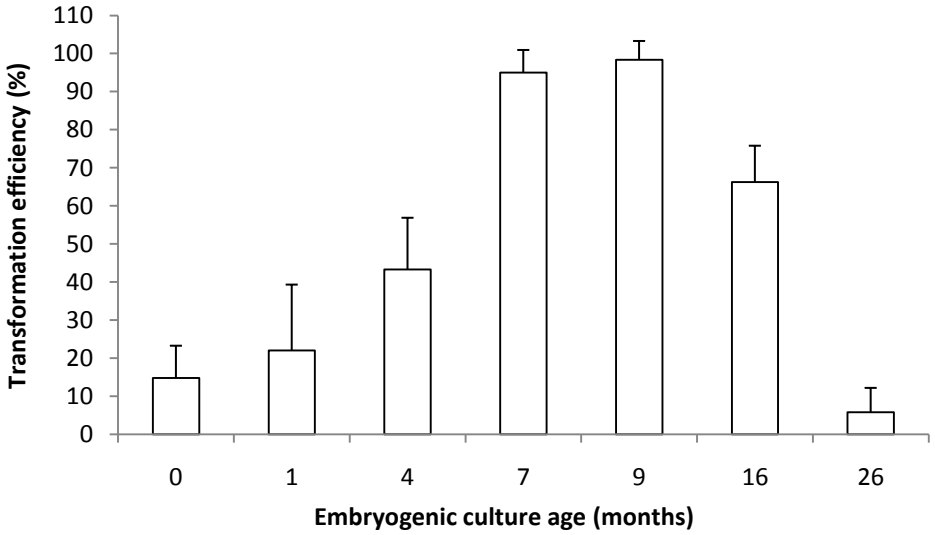

Figure IV. 5 Effect of the embryogenic callus culture age on *A. tumefaciens*-mediated transformation efficiency in *C. arabica*. Transformation efficiency was assayed 6 weeks after the end of co-cultivation by GFP epifluorescence. The transformation efficiency was analyzed by the proportion (p) of transformed calli (p=x/n), where x was the number of transformed calli and n the number of experiments. A 3 δ confidence limit for binomial distribution was calculated using the formula $p \pm 3\sqrt{p(1-p/n)}$ level of confidence 99%. All the transformation experiments were conducted independently in five to six replicates containing each 40 calli for each culture age (200 to 240 calli/culture age).

Histological studies revealed a change in the quality of embryogenic callus tissues depending on the culture age without any changes at the nude eye (Fig. IV.6). While the most homogeneous and active tissues were observed for 7 and 9-month-old embryogenic callus cultures that showed the typical histological aspect of the yellow phenotype (Fig. IV.6 B), the histological appearance of the primary embryogenic callus (no proliferation period) and old embryogenic callus cultures was very different. Observation of primary embryogenic callus indicated a very heterogeneous structure (Fig. IV.6 A) with coexisting degenerating and active areas. The cells exhibited a small nucleus and low starch and cytoplasm soluble protein contents. On a histological level, the old embryogenic cultures

were much more heterogeneous than at 7 months and appeared as a mix of isolated cells, pro-embryos and masses oriented towards somatic embryogenesis (Fig. IV.6 C). Other areas comprised cells with protein-rich cytoplasm similar to those from younger embryogenic cultures.

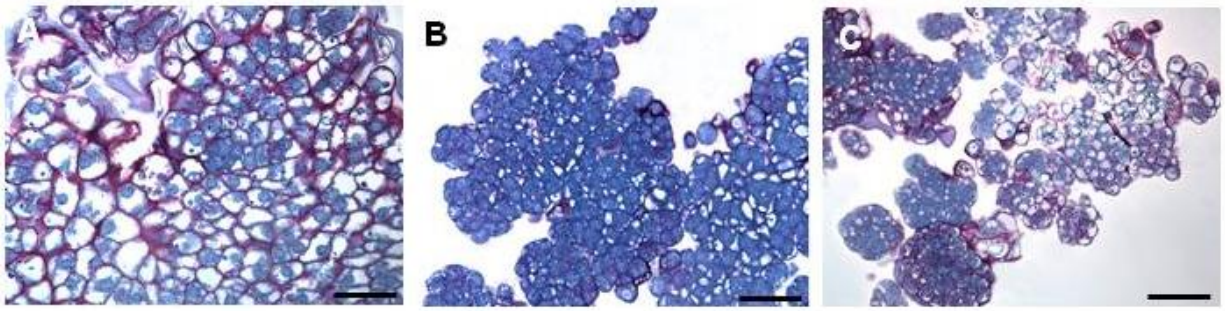

Figure IV. 6. Histological aspect of coffee embryogenic callus cultures depending on the age of the culture. Primary embryogenic callus produced on leaf explants; note the heterogeneous structure where degenerating (arrow) and active areas co-exist; the cells are characterized by a small and non-central nucleus and low starch and cytoplasm soluble protein contents, scale bar = 36.5 µM (A). Seven-month-old embryogenic callus culture; observe the highly homogeneous structure mainly comprising proembryogenic masses (PEMs) whose cells exhibit a high nucleus:cytoplasm ratio and dense cytoplasm rich in soluble and reserve proteins, scale bar = 73 µM (B). Twenty-six-month-old embryogenic callus culture; heterogeneous aspect of a mix of isolated cells, proembryos, masses oriented towards somatic embryogenesis (arrow), along with areas of cells with protein rich cytoplasm similar to those from younger embryogenic cultures, scale bar = 73 µM (C).

Transgenic coffee plant regeneration and molecular analysis

Transgenic plants were regenerated using 7-month-old embryogenic cultures with the yellow phenotype, according to the optimum proliferation and co-culture conditions established in this work. A total of 560 calli was co-cultivated. The transfer of embryogenic calli to hygromycin selection medium enhanced rapid browning (Fig. IV.7 A), but two months later a lot of resistant yellowish calli had grown on the surface of most of the necrotic calli (Fig. IV.7 B). After 4 months of hygromycin selection, of the 560 co-cultivated calli a total of 462 produced independent yellow resistant calli (transformation efficiency = 82.5%). All the non-transformed cultures (negative control) turned brown and died during the hygromycin selection period and neither yellow calli nor embryo regeneration could be observed. Each resistant callus line regenerated several putatively transformed somatic

embryos on the hygromycin-enriched medium (Fig. IV.7 C). Almost all resistant torpedo-shaped mature embryos germinated and developed whole plantlets (Figs. IV. 7D, E). Several thousand putatively transformed plants were regenerated. Among them a hundred and twenty plants from sixty independent transformation events (2 plants/transformation event), were chosen to be acclimatized in the greenhouse for further molecular controls (Fig. IV.7 E).

PCR analysis was conducted to detect the *HPTII* hygromycin resistance gene from the DNA of 60 putatively transformed plants derived from independent transformation events (1 plant/transformation event). Bands indicating the presence of the *HPTII* gene were systematically detected in the analyzed plants (Fig. IV.8) indicating the high efficacy of hygromycin selection. No band was observed in untransformed plants and in the blank negative control. Southern blot analysis was performed to determine the copy number of the *HPTII* gene integrated into the coffee genome (Fig. IV.9). Eleven plants from independent transformation events were analyzed. Five of them presented one transgene insertion (event n° 2, 3, 9, 10, 11). The other ones presented multiple copy insertion: two (n°5 and 7), four (n°6), 5 (n°8), 6 (n° 4) and 7 (n° 12).

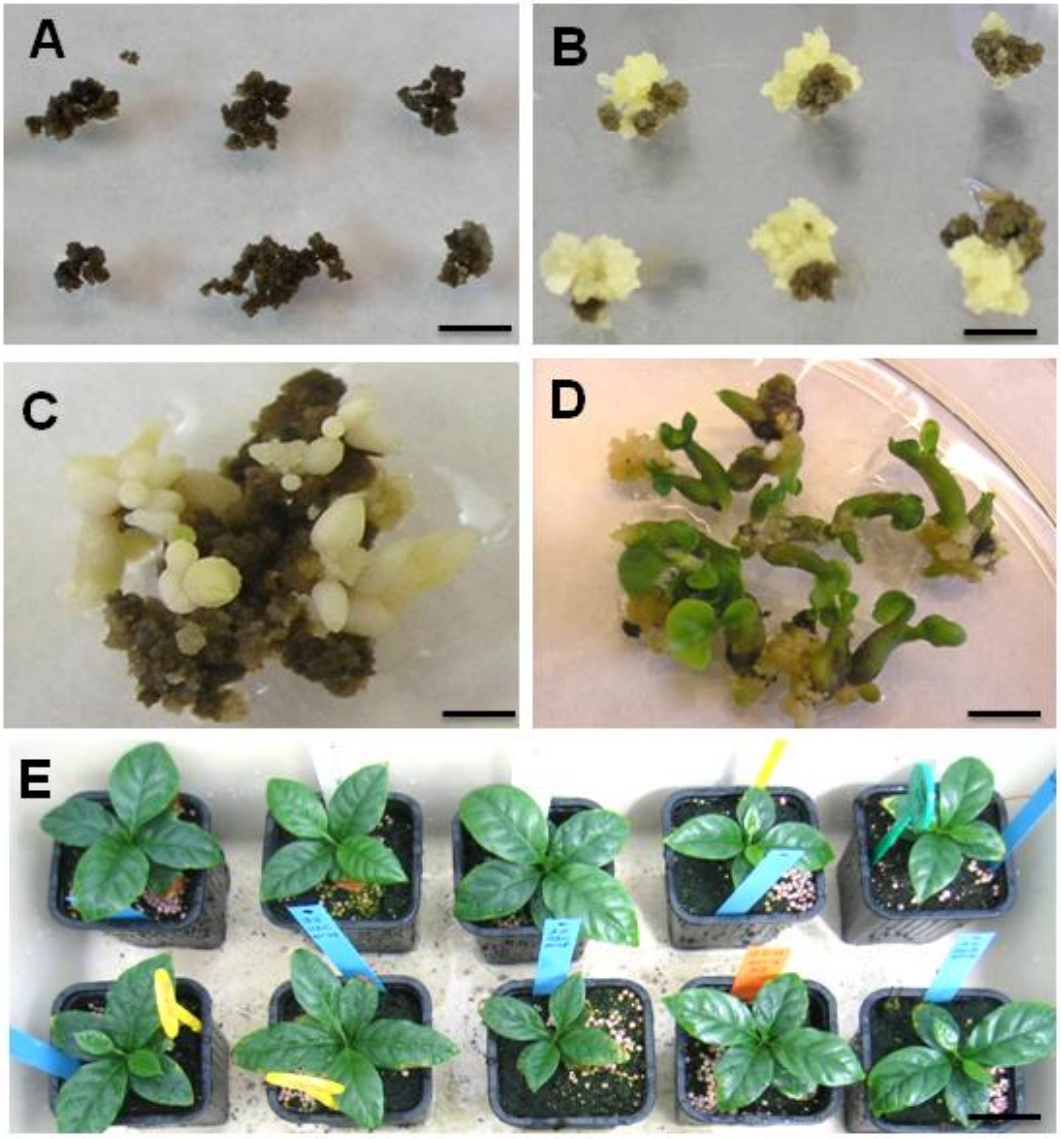

Figure IV. 7 Regeneration of transformed coffee plants from maintained embryogenic cultures. Calli in selective media containing 100 mg.L^{-1} hygromycin and 125 mg.L^{-1} cefotaxime, 4 months after co-cultivation with *A. tumefaciens* LBA1119 without plasmid, used as a control (A). Regeneration of resistant calli in selective media containing 100 mg.L^{-1} hygromycin and 125 mg.L^{-1} cefotaxime, 4 months after co-cultivation with *A. tumefaciens* LBA1119 carrying pMDC32; the yellow calli are resistant to hygromycin (B). Regeneration of torpedo-shaped somatic embryos 6 months after co-cultivation (C). *In vitro* plantlet development 8 months after co-cultivation (D). Acclimatization of transgenic plants in the greenhouse 12 months after co-cultivation (E).

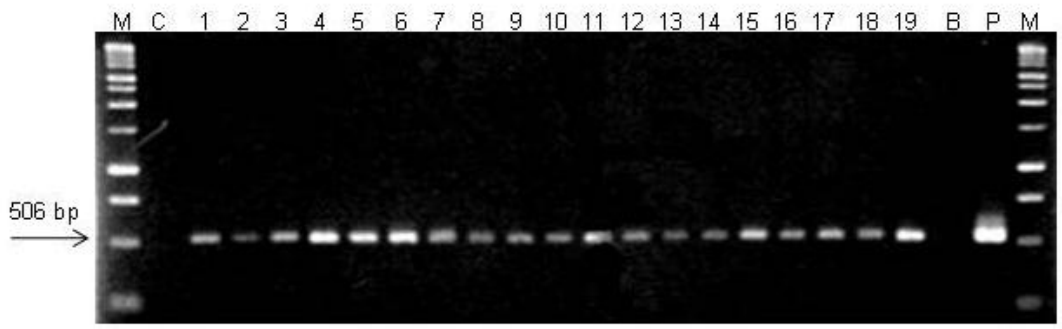

Figure IV. 8 PCR detection of the hygromycin gene in transgenic coffee plants. The plants were produced from 7-month-old embryogenic callus cultures co-cultivated with *A. tumefaciens* strain LBA1119 carrying out the pMDC32 binary vector. M - Molecular weight DNA markers (1kb), C – untransformed coffee plant (control), *1–19* transgenic coffee plants derived from independent transformation events , B – blank, PCR mix without DNA, P – plasmid. Arrow indicate fragment correspond to hygromycin gene (506 bp).

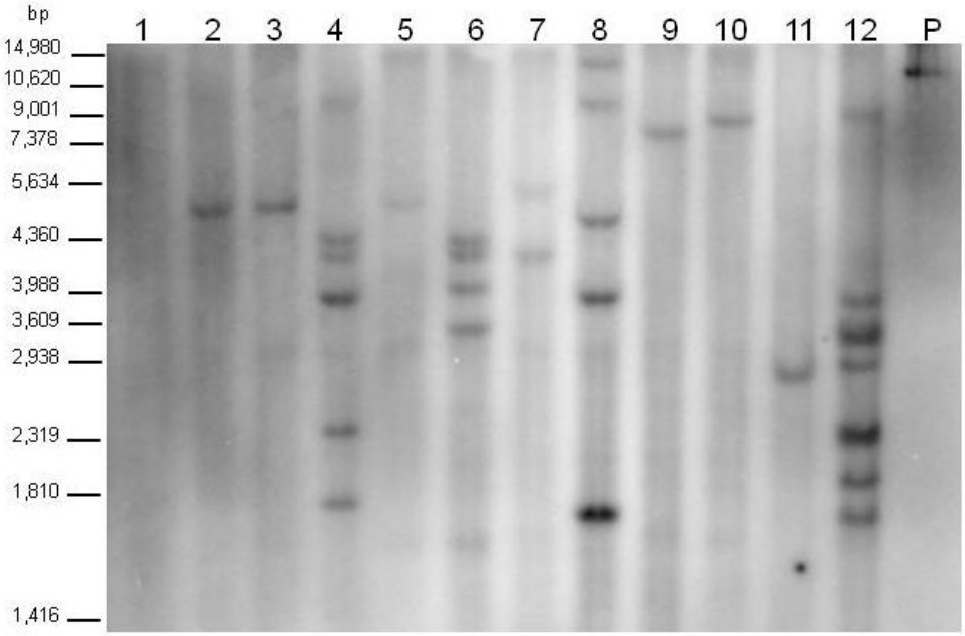

Figure IV. 9 Southern blot analyses of transgenic coffee plants. Coffee DNA from leaf material was digested with *Eco*RI, electrophoresed and probed with a ^{32}P labeled HTPII gene fragment. The number of bands reflects the number of transgene insertions. Lane 1, untransformed coffee (control). Lanes 2-12, transgenic plants from independent transformation events. P - plasmid control (pMDC32). The markers' sizes are indicated on the left.

DISCUSSION

Over the last ten years, embryogenic callus directly produced by leaf explants has been the most used tissue for *Agrobacterium*-mediated transformation in coffee (Etienne et al., 2008). Nevertheless, as well as for other perennial crops, the availability of embryogenic tissues suitable for genetic transformation remains one of the main bottlenecks for developing genetic transformation strategies. In this work, we established for the first time the conditions for long-term proliferation of embryogenic cultures in the *C. arabica* species and described a highly efficient and reliable *Agrobacterium*-mediated transformation method based on the use of these embryogenic cultures.

Stable transformation was efficiently monitored using the GFP reporter gene. The fluorescent marker GFP has the main advantage compared to other reporter genes that it enables non-invasive detection of transformed cells without the introduction of co-factors or destruction of the biological sample (Chalfie et al., 1994). Similarly, GFP visual selection was recently used as the sole marker to detect transgenic calli lines of *Hevea brasiliensis* without antibiotic pressure (Leclercq et al., 2010). Using long-term embryogenic callus cultures to transform Arabica coffee proved to be very efficient and, under the best conditions, stable expression of the GFP gene was observed in up to 95% of the tested calli. In previous work on coffee, transformation efficiency ranged from less than 0.1% for the recalcitrant *C. arabica* (Leroy et al., 2000) to 33% for *C. canephora* (Canche-Moo et al., 2006). The reliability of detecting GFP fluorescence to monitor transformation success in coffee was confirmed by a large-scale trial aimed at regenerating fully-transformed plantlets using the hygromycin marker gene with which similar transformation efficiencies (82.5%) were obtained. As previously reported (Mishra et al., 2002; Ogita et al., 2004), our study showed that the 50-100 $mg.l^{-1}$ hygromycin rate is highly efficient in selecting transformed cells as no escapes were observed.

Parameters already known to affect coffee transformation were evaluated using long-term embryogenic callus cultures as explants. In the majority of studies on *A. tumefaciens*-mediated transformation in the *C. arabica* species, the co-culture temperature used was 25-26°C (Hatanaka et al., 1999; Ogita et al., 2004). We demonstrated here that a reduction to 20°C increased transformation efficiency. A cooler co-cultivation temperature (15-18°C) also increased the efficiency of *A. rhizogenes*-mediated transformation in the same species (Alpizar et al., 2006). Fullner and Nester (Fullner and Nester, 1996) showed that temperatures above 29°C affected *Agrobacterium* T-DNA machinery transfer and tumour formation did not occur at this temperature and the best co-culture temperature was around 22°C. The same authors (Fullner et al., 1996) showed that 19°C was the optimum temperature for a pilus assembly and T-DNA transfer.

The positive effect on genetic transformation of reducing the strength of mineral salts in the pre-culture medium is consistent with the literature. Although it has been largely demonstrated that ions are involved in bacterial attachment to plants (Romantschuk, 1992), transformation has been

successfully achieved in numerous species by using half-strength or more diluted salt solution media or solutions specifically lacking certain salts such as $CaCl_2$ in the pre-culture medium (Montoro et al., 2000) or co-cultivation medium (Azadi et al., 2010; Cheng et al., 1997). The role of growth regulators on transformation has also been reported.(Blanc et al., 2006) showed that simultaneously increasing the auxin and cytokinin supply in the pre-culture medium prior to transformation stimulated the development of active and fast growing cells, hence transformation efficiency. It has been shown during long-term culture that cultures may lose the need for auxin and/or cytokinin to maintain active growth. This process known as 'habituation', which is common in callus cultures from some plant species such as sugar beet (Van Geyt and Jacobs, 1985), was described as a shift from auxo- to autotrophic state for growth regulator requirement (Meins and Foster, 1985). Coffee embryogenic callus cultures effectively do not require auxin or cytokinin to proliferate, though it was possible to stimulate it by optimum exogenous concentrations of both growth regulators. Similarly, genetic transformation of long-term maintained embryogenic cultures was possible without auxin or cytokinin supply. Our results specifically showed the very negative role of a cytokinin supply and the positive role of auxins in acquiring transformation ability.

To establish long-term embryogenic callus cultures in coffee, active embryogenic tissues have to be sub-cultured every 4 weeks on the proliferation medium. It is well accepted that a lack of regular sub-cultures leads embryogenic calli to lose their embryogenic potential as a result of cell ageing. Sub-cultures were also used to homogenize the culture by selecting recently formed active embryogenic tissues. Sub-culturing every 14-21 days on a maintenance (i.e. proliferation) medium is an essential step in establishing long-term cultures in many perennial crops such as mango (Ara et al., 2005), cacao (Maximova et al., 2005), *Pinus patula* (Ford et al., 2005), *P. taeda* (Tang and Newton, 2005), *Picea mariana* (Tremblay et al., 2005), *P. radiate* (Walters et al., 2005) and other forest trees (Jain, 2006). The process of establishing coffee embryogenic cultures leads to the development and maintenance of three morphologically different callus phenotypes with highly contrasting potentials for genetic transformation. We demonstrated that the yellow type is an ideal material for transformation purposes (almost 100% transformation efficiency) while the grey and white ones were totally recalcitrant. Several morphological variants have also been observed during the proliferation and maintenance of embryogenic calli from cotton (Wu et al., 2008). The authors showed that the transformation efficiency of fine-grained yellow or light yellow embryogenic callus was much higher than the coarse-grained grey or brown callus, although no histological or biochemical characterization was carried out to explain such differences.

A priority of any team involved in developing a transformation procedure is to identify the suitable target cell type. On a histological level, the yellow coffee callus with transformation ability proved to merely consist of PEMs that were proliferating compact cell masses able to produce somatic embryos and consisted of small, very dense cytoplasm embryogenic cells similar to those observed in

the *Dacus carota* model (Ibaraki and Kurata, 2001; Pierik, 1997). Only observation of the yellow phenotype guarantees the presence of such competent PEMs, hence successful transformation, insofar as the maintained embryogenic cultures are not too old (< 1.5 years). In the case of primary embryogenic callus and of old yellow callus embryogenic cultures, decrease of transformation efficiencies was systematically accompanied by a marked reduction in the presence of PEMs.

Moreover, our study showed that the positive effect of an auxin balance on the embryogenic callus transformation potential was also accompanied histologically by an increase in PEM presence (data not shown). Taken together, these results indicate that PEMs are probably the competent tissue for coffee genetic transformation. PEMs have been used previously for genetic transformation of other woody species including *Vitis vinifera* (Mauro et al., 1995), *V. rotundifolia* (Dhekney et al., 2008) and American chestnust (Andrade et al., 2009). It is interesting that, although embryogenic tissue is the most used tissue for coffee transformation, relatively weak transformation efficiencies were obtained with primary embryogenic callus directly regenerated on leaf explants. The histological heterogeneity of coffee primary embryogenic calli was previously reported (Söndahl et al., 1979). Our work confirmed the strong heterogeneity of this tissue but also revealed its low transformation-competent PEM contents. Consequently, we recommend avoiding direct use of primary embryogenic callus for transformation experiments and the completion of some proliferation cycles to allow the selection of PEMs and consequently drastically increase transformation potential with a view to routine functional analysis.

The reasons for the high genetic transformation ability of PEMs present in maintained coffee embryogenic cultures could be morphological and/or epigenetic. The particular structure of this cell type could favorably influence the *Agrobacterium*-mediated transformation process. For instance, (Sangwan et al., 1992) showed in *Arabidopsis thaliana* that, irrespective of their origin, the competent cells were small, isodiametric with thin primary cell walls, small vacuoles, prominent nuclei and dense cytoplasm. Most of these characteristics correspond to those of coffee PEMs. The small size of PEMs associated with their looser organization increases bacterial accessibility. In addition, the high regenerative potential of this cell type is an indispensable quality for regenerating transgenic plants. Furthermore, genetic and epigenetic changes occur during long-term cultures of rapidly dividing dedifferentiated cells. Mechanisms such as changes in DNA methylation, reactivation of transposable elements, histone modifications or siRNA biogenesis have been recently described (Tanurdznic et al., 2008; Valledor et al., 2007). Hormone habituation and somaclonal variations in long-term embryogenic cultures – which are two manifestations of epigenetic regulation (Meins and Foster, 1985; Valledor et al., 2007) – were reported in coffee in the present study and in an earlier one (Etienne and Bertrand, 2003), respectively. It was shown that the auxin 2,4-dichlorophenoxyacetic acid (2,4-D) maintains an undifferentiated state and habituation to *in vitro* proliferation through increased DNA methylation (Causevic et al., 2005; Von Aderkas and Bonga, 2000). Our results

highlighted that a high 2,4-D:6-BA ratio in the proliferation medium gave successful genetic transformation. It could therefore be suggested that this stimulation may have resulted from DNA hypermethylation.

CONCLUSIONS

Our work shows that obtaining and monitoring long-term maintained embryogenic cultures is a reliable way of producing transformed plants in a recalcitrant woody species. The transformation process set up in the present study undoubtedly benefited from the well-established regeneration protocols developed for the commercial application of *C. arabica* somatic embryogenesis to the mass distribution of elite heterozygous clones (Bertrand et al., 2010; Menéndez-Yuffá et al., 2010) and from the profound knowledge of cell characteristics and cellular mechanisms involved in embryogenic tissues and developing embryos. It is noteworthy in our study that most of the progress obtained in increasing the transformation efficiency was achieved by optimizing the production conditions of the embryogenic tissues used for transformation experiments much more significantly than with conventional optimization of the physical co-cultivation conditions. The establishment of maintained embryogenic cultures and optimization of their physiological status could be a valuable way of establishing reliable transformation conditions in other recalcitrant woody species in view of high-throughput functional analysis of genes.

METHODS

Plant material

All the studies were conducted using the genotype *C. arabica* var. Caturra. The leaf explants were collected from trees maintained in a greenhouse located at IRD (Montpellier, France) and the seeds came from Finca Santa Edwige (Alajuela, Costa Rica). The induction of embryogenic calli was carried out as previously described (Etienne, 2005). Briefly, 1 cm^2 pieces of young leaves from the mother-tree were surface-sterilized and used as explants. Leaves were disinfected by immersion in 10% calcium hypochlorite solution containing 1% Tween 80 for 20 min followed by 8% calcium hypochlorite solution for 10 min and rinsed 4 times with sterile water. The explants were cultured for 1 month on MS/2 (Murashige and Skoog, 1962) 'C' callogenesis medium containing 2.26 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 4.92 μM indole-3-butyric acid (IBA) and 9.84 μM iso-

pentenyladenine (iP). The explants were then transferred for 6-8 months to MS/2 'ECP' embryogenic callus production medium containing 4.52 µM 2,4-D and 17.76 µM 6-benzylaminopurine (6-BA). Yellow or whitish primary embryogenic callus appeared on the initial necrotic callus. Primary embryogenic callus was induced in baby food jars at 27°C in the dark. All the media used in this work were supplemented with 30 g/L sucrose and had the pH adjusted to 5.7 prior to the addition of 2.8 g/L phytagel. The media were autoclaved at 120°C and 1.1 kg/cm^2 for 20 min, and then 25 ml of medium was dispensed into each 100 x 15 mm Petri dish or baby food jar.

Establishment of an embryogenic cell suspension

Primary embryogenic calli were transferred to 250 ml Erlenmeyer flasks at a density of 1 g/L in MS/2 'CP' liquid proliferation medium [78] with 4.52 µM 2.4-D and 4.65 µM kinetin, and shaken at 100 rpm in the dark at 27°C. Cell suspension cultures of embryogenic aggregates were generally established after 3 months under such conditions.

Zygotic embryo extraction

Sterilization was carried out by immersing the seed in 8% HClO bleach solution. The seeds were stirred for 5 min., then submitted to a vacuum for 20 min and stirred again for 5 min. They were finally rinsed 3 times in sterile water, and soaked in Petri dishes containing sterile water (2 cm deep) placed in the dark at 27°C. Under these conditions, the seeds were totally imbibed after 48-72h. The embryos were extracted after removing the endosperm and directly placed under co-cultivation conditions after injury with a scalpel blade.

Establishment of maintained embryogenic callus cultures

Long-term embryogenic cultures were successfully established using the primary embryogenic callus and by the monthly transfer of yellowish fragments collected from the upper part of embryogenic calli to fresh semi-solid 'ECP' MS/2 embryogenic callus production medium [78] with 4.5µM 2.4D and 12µM 6-BA solidified with 2.8 g/L phytagel. The cultures were placed in baby food jars at 27°C in the dark. To optimize the proliferation conditions, the following modifications to the ECP medium were compared by assessing the subsequent callus growth and transformation efficiency after 3 sub-cultures: auxin concentration (0, 1.8, 4.5, 9 and 18 µM 2.4D), cytokinin concentration (0,

3, 13, 23 and 32 µM 6-BA) and Macro and Microelement salt concentration (MS/4, MS/2, MS, 1.5MS). Moreover, in order to evaluate the importance of callus morphology and culture ageing, different embryogenic callus phenotypes observed under the same culture conditions [yellow, whitish and grey (Fig. IV.1)] and embryogenic cultures of different ages (primary embryogenic callus or 1, 4, 7, 9, 16 and 26-month-old embryogenic callus cultures exhibiting the same yellow phenotype) were compared for subsequent transformation efficiency.

***Agrobacterium* strain and binary vector**

The disarmed strain of *A. tumefaciens* LBA1119 carrying a binary vector (pBIN35SGFP) containing the reporter gene *GFP5* coding for green fluorescent protein under control of the constitutive cauliflower mosaic virus (CaMV) 35S promoter was used for all experiments aimed at establishing a transformation protocol. The LBA1119 strain carrying the binary vector pMDC32 (Curtis and Grossniklaus, 2003) containing the HPTII gene conferring hygromycin resistance was used to regenerate transgenic plants and validate the transformation protocol using the same culture conditions.

Co-cultivation and decontamination

Coffee explants were maintained in their culture container, i.e. baby food jars, and immersed in 10 ml of *A. tumefaciens* suspension (OD_{600}=0.6) for 10 min without shaking. The suspension was removed and the inoculated explants were co-cultivated at 27 or 20°C for 5 days in the dark. After this period, the explants were rinsed twice with 20 ml sterile water, and then 20 ml of ECP medium containing 1.2 g/L cefotaxime was added to each jar. The cultures were placed on a rotary shaker at 30 rpm for 3 hours. After that time, the liquid was removed and the calli rinsed with ECP medium for 15 min. The liquid was removed and the explants were blotted on dry filter paper to remove excess bacterial solution. They were subsequently placed in Petri dishes containing ECP medium with 500 mg/L cefotaxime. From each jar, 4 Petri dishes were made up containing 20 explants each. The cultures were placed in the dark for 4 weeks at 27°C and then observed to assess GFP reporter gene activity. To optimize the co-cultivation conditions, different explants (zygotic embryo fragments, 4-month-old embryogenic cell suspensions and 4-month-old embryogenic callus cultures), *Agrobacterium* suspension concentrations (undiluted OD_{600}=0.6) or diluted to 1/10 by adding 'ECP' medium) and temperatures (20 and 27°C) were compared by assessing subsequent transformation efficiency. The last two parameters were tested on four-month-old embryogenic callus cultures.

Evaluation of transformation efficiency by GFP visualization

All the tissues tested for genetic transformation were used to evaluate stable expression of *GFP* 30 days after *Agrobacterium* inoculation. Plants were screened for GFP expression using a Fluo III fluorescence microscope (Leica) with GFP1 (excitation filter 425 nm; emission filter 480 nm), GFP2 filters (excitation 480 nm; emission 510 nm) and under green fluorescence (excitation filter 546 nm; emission filter 590 nm). The autofluorescence from chlorophyll was blocked using a red interference filter. Transformation efficiency was calculated as the proportion (p) of transformed explants (p=x/n), where x was the number of transformed explants exhibiting GFP and n the number of experiments.

Histological observations

The samples were fixed for 24 h in a solution containing 1% glutaraldehyde, 2% paraformaldehyde and 1% caffeine in a 0.2 mM phosphate buffer at pH 7.2. After this, they were dehydrated in a graded series of ethanol, embedded in a 7100 resin (LKB) and cut into 3 µm longitudinal sections. The sections were double-stained with PAS (periodic acid-Schiff)-NBB (naphthol blue black). PAS specifically stains polysaccharides red (walls and starch) and NBB stains soluble and insoluble proteins blue (Fisher, 1968; Buffard-Morel et al., 1992). Sections were observed by conventional light microscopy through a DM600 Leica microscope and photographed. Two magnification lenses were used: Lens 109 Numerical aperture = 0.30 HC PL Fluotar (Ref. Leica 11506505) and Lens 209 Numerical aperture = 0.70 HC Plan APO (Ref. Leica 1506166). Pictures were taken with a Retiga 2000R camera (GImaging Co.).

Regeneration of transgenic plants

The best culture conditions were applied in a large-scale experiment to transform 560 calli from 7-month-old embryogenic cultures using the LBA1119 strain carrying the binary vector pMDC32. After decontamination, the cultures were subcultured every 4 weeks twice on 'R' regeneration medium (Etienne, 2005) containing 17.76 µM 6-BA and 50 mg/L hygromycin and decreasing cefotaxime concentrations (250, 125 $\mu g.ml^{-1}$) and twice on 'M' maturation medium containing 1.35 µM 6-BA, 100 mg/L hygromicin and 125 mg/L cefotaxime. The other subcultures were carried out on 'M' maturation medium containing 1.35 µM 6-BA devoid of cefotaxime and hygromicin until plantlet development. Several plants regenerated from each resistant callus. Ten months after co-cultivation, the plantlets were acclimatized in the greenhouse. During the entire

regeneration process, the cultures were maintained under a 14 h photoperiod (20 µmol.$m^{-2}s^{-1}$ light intensity) at 26°C until acclimatization.

PCR and Southern blot analysis of transgenic plants

Sixty putatively transformed greenhouse plants derived from independent transformation events (1 plant taken at random/transformation event) and produced in the large-scale experiment were subjected to PCR analysis to detect the presence of the hygromycin resistance gene. The leaves were frozen in liquid nitrogen and DNA extraction was carried out with a CTAB procedure (Doyle and Doyle, 1987) with modified extraction buffer (2% CTAB, 1.4mM NaCl, 100mM Tris HCl, 20mM EDTA pH 0.8). The primers used for amplification of a fragment of HPTII gene from the pMDC32 vector were: 5'-GCCTGAACTCACCGCGACGTC-3' and 5'-GCACTGACGGTGTCGTCCAT-3' (fragment size 506 bp). PCR was carried out in a 25 µl volume containing 50 ng of leaf genomic DNA, 1U Taq DNA polymerase, 1X buffer (Promega, Madison USA), 0.2 mM each dNTP, 2mM $MgCl_2$, 0.2 µM each primer. Amplification was performed in thermal cycler GeneAmp® PCR system 9700, Applied Biosystem) in the following reaction: 1 cycle of 2min 95°C, followed by 30 cycles 1 min at 95°C, 1 min 55°C, 1 min 72°C and 4 min 72°C of final extension. The reaction products were electrophoresed in 1% (w/v) agarose gels and visualized by staining with ethidium bromide.

Among the PCR-positive plants, 11 derived from independent transformation events were chosen to perform Southern blot analysis to determine the number of transgenes integrated into the genome. Briefly, 20 µg of genomic DNA was digested with the restriction endonuclease *Eco*RI. DNA fragment separation by electrophoresis, transfer to nylon membrane, radioactive probe labeling and hybridization were performed as previously reported (Noir et al., 2004).

Data processing

Transformation efficiency was calculated as the proportion (p) of transformed explants (p=x/n), where x was the number of transformed explants exhibiting GFP and n the number of experiments. A 3 δ confidence limit for binomial distribution was calculated using the formula $p \pm 3\sqrt{p(1-p/n)}$ level of confidence 99%. All the transformation experiments were conducted independently in three or four replicates. Callus growth was measured by the difference between the final and initial weight of embryogenic cultures after a 4-week proliferation cycle. The initial weight was calibrated at 120 ± 10 mg. Each data corresponds to a mean ± SD from 4 measurements. We performed an ANOVA followed by the Tukey HSD test to determine the significant differences between the means of all treatments.

CHAPTER V

IDENTIFICATION AND FUNCTIONAL VALIDATION OF CANDIDATE GENES TO COFFEE LEAF RUST RESISTANCE (CLR)

This chapter is subdivised in three parts. In part I, it describes the approaches used for estimation of the number of candidate genes (CG) to CLR resistance in the resistant cultivar. The part II, presents the strategy used for cloning the candidate genes while the developed procedure for functional validation of the CG in transgenic coffee plants are described Part III.

Identification and functional validation of candidate genes to CLR

INTRODUCTION

The S_H3 resistance factor against the coffee leaf rust (CLR)(*Hemileia vastatrix*) was originally identified into *C. liberica* species (2n=2x=22) (Wagner & Bettencourt, 1965). A natural coffee hybrid between *C. arabica* and *C. liberica* discovered in India (called S.26) (Vishveshwara, 1974; Sreenivasan et al., 1993) and carrying the S_H3 resistance factor has been used in *C. arabica* (2n=4x=44) breeding programs in India as main source of rust resistance.

The segregation analysis of an F_2 population derived from a cross between a susceptible and a *liberica*-introgressed accession was essayed against three different races of *H. vastatrix*. The analysis revealed that the S_H3 gene segregated at 3:1 ratio, as expected for a single dominant gene (Prakash et al., 2004). The *liberica* introgressed-fragment carrying the S_H3 resistance gene wass localized in a distal position in the *C. arabica* chromosome 1 (Herrera et al., 2007). Several molecular markers were found linked to the resistance (Chapter II).

Sequence-characterized amplified region (SCAR) markers associated with S_H3 gene were developed (Mahé et al., 2008) and used to screen a bacterial artificial chromosome (BAC) library from *C. arabica* cv. IAPAR 59 (Noir et al., 2004) to generate a physical map of S_H3 locus (Chapter II). Although *C. arabica* cv. IAPAR 59 is resistant to coffee leaf rust, this cultivar does not have S_H3 resistant factor but contains other unidentified resistance gene(s). Several BAC clones of this region were identified. The analysis of BAC sequences revealed eight members of a putative R gene family that are present in two homoelogous loci and are distributed in two regions (hereafter indicated as A and B) separated by more than 160 kb. The subgenome E^a has 3 copies in region A and 2 copies in region B whereas subgenome C^a contains 2 copies in region A and 1 copy in region B. The identified R gene family belongs to the CNL subclass (hereafter S_H3-CNL family) (Chapter III). No other putative R genes were detected in the S_H3 region.

A ‘Candidate gene approach’ was used to try cloning the S_H3 genes from the *C. arabica* S.795 accession (derivate of S.26 hybrid) containing the *C. liberica* introgression.

PART I.

Estimate of *S_H3*-CNL gene members in the resistant cultivar

The first question to be answered was how many members of the *S_H3*-CNL family exist in the *S_H3* introgressed region of *C. arabica* S.795 resistant accession? Two approaches were essayed: Southern blot and sequence analysis.

a) Estimate of the number of the *S_H3*-CNL members by Southern blot analysis

This approach is based on the comparative analysis of *S_H3*-CNL specific hybridization patterns of introgressed and non introgressed accessions.

Material and Methods

Total DNA from the introgressed accession (S.795) and from two non introgressed cultivars (IAPAR 59 and Bourbon) was isolated from leaves using a CTAB procedure (Doyle and Doyle 1990) with modified extraction buffer (4% CTAB, 1.4mM NaCl, 100mM Tris HCl, 20mM EDTA pH 0.8).

Genomic DNA was digested with *Dra*I, *Eco*RI or *Bam*HI restriction enzymes. The restriction fragments were separated by electrophoresis and transferred on to Hybond-N^+ (Amersham Biosciences) nylon membranes for Southern hybridizations. *S_H3*-CNL family specific probe was generated by PCR amplification using primers designed on the NBS domain (left primer: 5'-CGGTCTCGGTAAGACCACTC-3'and right primer 5'-CCTCTGCAAATGGAAATGCT-3'). The obtained 516 bp fragment was labeled with ^{32}P-ATP and used as probe with hybridization stringent conditions (Sambrok et al., 1989).

Result and Discussion

The expected sizes of the eight restriction fragments containing the probe target on IAPAR-59 was determined by an *in silico* restriction analysis with *Dra*I, *Eco*RI or *Bam*HI on the BAC sequences containing the *S_H3*-CNL members table V.1. Except one of all expected fragments were identified on the respective hybridization patterns; due to the size, the *Bam*HI restriction fragment corresponding to

the B_C[a] member (51622 bp) comigrated with the high molecular weight fragments and was not detectable. None additional band was detected in the hybridization patterns of IAPAR-59, suggesting that this family is exclusively present at the *S_H3* locus in this cultivar. This result is consistent with previous data obtained by BAC filter hybridization using the *S_H3*-CNL family specific probe. In this experiment all positive BAC clones from the IAPAR BAC library appeared to belong to the *S_H3* region.

Table V. 1 *In silico* restriction analysis on the BAC sequences containing the *S_H3*-CNL members

S_H3-CNL members	*Dra*I	*Bam*HI	*Eco*RI
	Fragment size (bp)		
A1_E[a]	4800	3836	1840
A2_E[a]	3927	5685	3821
A3_E[a]	3686	7044	3109
B1_E[a]	5576	5491	4899
B2_Ea	6287	8815	3901
A1_C[a]	4804	3815	3013
A2_C[a]	3753	5387	4214
B_C[a]	5070	51622	5270

The comparative analysis of the hybridization patterns of the introgressed and non introgressed cultivar showed that the three IAPAR59 restriction fragments containing the C[a] copies of the R gene family (blue arrows in figure V.1) were lacking in the introgressed accession and at least four additional fragments were present in the S.795 (red arrows). These observations confirmed that the *C. liberica* introgression replaced the homoeologous C[a] region of *C. arabica* as previously suggested (Chapter 2). In the *Eco*RI restriction pattern five additional bands were observed in the S.795 hybridization pattern (Figure V.1). To date it is not possible establish if this observation is the result of a restriction site internal to the target sequence or it is an additional member of the *S_H3*-CNL family.

To a better comprehension of the southern hybridization interpretation a schematic representation of the patterns obtained with *Eco*RI enzyme is proposed in figure V.2. In this schema the fragments corresponding to the sub genome E[a] are represented in black while the sub genome C[a] and the introgression from *C. liberica* are shown as blue and red fragments, respectively. The E[a]+C[a] and E[a]+L represent the hybridization of IAPAR-59 and S.795 cultivar, respectively.

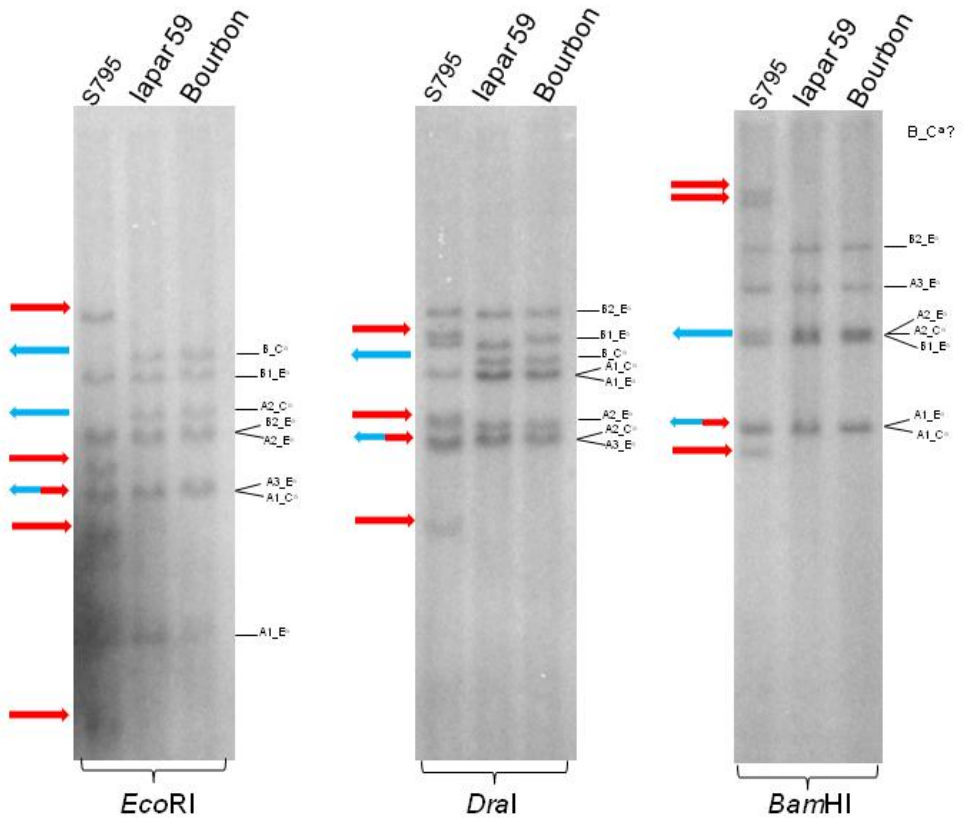

Figure V. 1 Southern blot patterns of *C. arabica* cultivars hybridized with S_H3-CNL specific probe. Total DNA was digested with *Eco*RI DraI and BamHI. Lane 1: S.795 (a *liberica*-introgressed accession that contains the S_H3 resistance factors), lane 2 and 3: IAPAR 59 and Bourbon, respectively (non-introgressed accessions). The blue arrows indicate the restriction fragments of non introgressed accessions missing in the S.795 accession; the red arrows indicate the restriction fragments originating from the *C. liberica* introgression the S.795 accession.

The copy number estimation based on southern hybridization has some limitations. First, the detection of additional bands into hybridization patterns obtained with given restriction enzyme could be complicated by superposition of different hybridization signals of fragments with very similar sizes, as observed for the IAPAR59 patterns, where co migrating fragments produced bands with stronger hybridization signal (e.g., in figure V.1, the DraI pattern, A1_C^a was not separated from A1_E^a, as well as A2_C^a co-migrate with A3_E^a). Second, the hybridization signal of restriction fragments with very large size could not be separated from the load of high molecular weight fragments; For instance, in the *Bam*HI hybridization pattern, the fragment corresponding to the B_C^a member (51622 bp) was not distinguishable (Figure V.1). Finally, partial gene duplication or gene remnants containing the probe target could be accounted as member of this family.

The additional hybridization bands observed in the pattern of the S.795 introgressed cultivar (red arrows in figure V.1) are thought to represent putative candidate genes for the S_H3 resistance factor.

To a better comprehension of the southern hybridization interpretation a schematic representation of the patterns obtained with *Eco*RI enzyme is proposed in figure V.2. In this schema the fragments corresponding to the sub genome E^a are represented in black while the sub genome C^a and the introgression from *C. liberica* are shown as blue and red fragments, respectively. The E^a+C^a and E^a+L represent the hybridization of IAPAR-59 and S.795 cultivar, respectively.

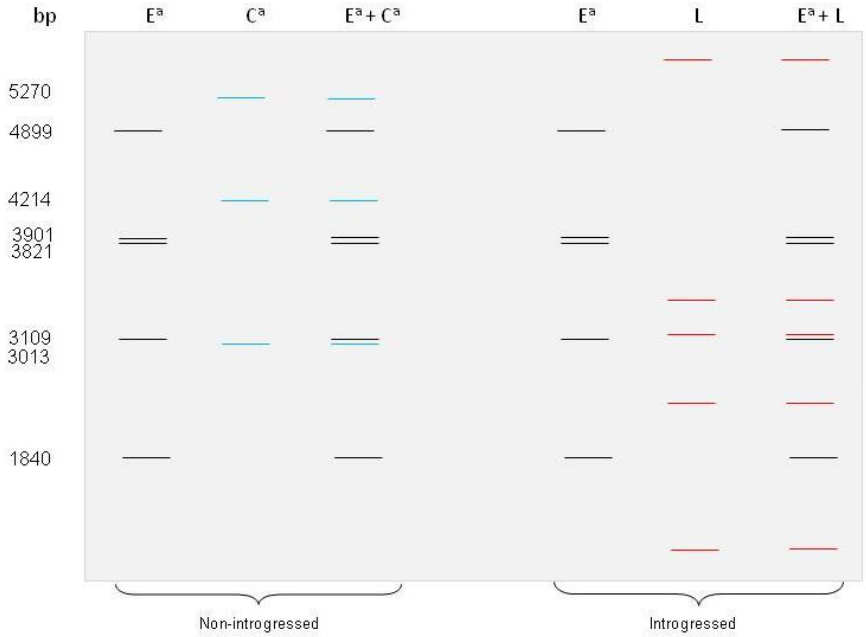

Figure V. 2 Schematic representation of hybridization pattern obtained with *Eco*RI in non-introgressed and introgressed acession of *C. arabica*. The fragment sizes are on the left. The black and blue and bands correspond to the hybridization fragments of non introgressed accessions assigned to the E^a and C^a sub genomes, respectively. The red bands correspond to the *C. liberica* introgressed fragment on the S.795 accession.

b) Estimate of the number of the S_H3-CNL members by sequence analysis

This approach is based on the discrimination of the S_H3-CNL family members by sequence polymorphism, i.e. single nucleotide polymorphism (SNP) and insertion/deletion (INDEL). Since introns are expected to accumulate more mutations than coding regions (which are subject to natural purifying selection), their sequences should have more useful polymorphism to discriminate the different copies of the S_H3-CNL family in S.795 cultivar. Consequently, based on the S_H3-CNL member sequences, primers were designed on exon 1 and 2 to allow the amplification of a region containing the intron of each known S_H3-CNL member.

Material and Methods

A pair of primers was designed to amplify a gene portion containing the intron of the *S_H3*-CNL family members, Left: 5'- GAGACCTTCTGAAGCAGCTTGT-3' and right 5'-ACTCAGAGCCAACACCTGCAAC-3'. In order to minimize the false polymorphism introduced by polymerase error, DNA amplification was performed with High fidelity Platinum kit (Invitrogen Carlsbad, CA). The mixes were put together and amplified using Biorad Thermal cycler. PCR conditions was as follow: 94°C 4 min followed by 30 cycles of 1 min 94°C, 1 min 55°C, 4 min 68°C and final extension of 7 min 68°C. In order to verify the PCR result, a 10µl aliquot from each amplification was analyzed by 1% agarose gel electrophoresis.

The PCR amplicons were cloned into pCR®2.1-TOPO® kit from Invitrogen and inserted into *E. coli*-TOP10 chemically competent cells according to the manufacturer's protocol. Plasmid DNA was isolated using Promega Wizard® *Plus* Minipreps DNA purification System (Promega Corporation, Madison, WI, USA) according to manufacture instruction. Three independent PCR and cloning were carried out and 32 clones from each PCR were sequenced using M13-universal-reverse primers. The experiment was repeated twice. A total of 192 clones were sequenced and analyzed using Staden and Bioedit software (Hall, T.A. 1999).

Results and Discussion

Based on sequence polymorphisms (INDELs and SNPs), at least ten different *S_H3*-CNL sequences were identified in the introgressed accession (S.795). Five of them clearly correspond to the members of the E^a sub genome. The other five sequences were considered originate from the *C. liberica* introgressed region. Again, these results confirmed that the introgression of *C. liberica* fragment replaced the homoeologous C^a region of *C. arabica*.

Furthermore, this approach could underestimate the *S_H3*-CNL member number: in fact, PCR could fail to amplify some *S_H3*-CNL family member due to eventual sequence mutations that prevent the primer annealing. On the other hand, the copy number could be overestimated due to the presence of partial gene duplications or gene remnants containing the target for the primers.

Conclusion

Although, the two approaches described above have some limitation, both strategies provided similar estimate number of *S_H3*-CNL members present in *liberica*-introgressed region (4 or 5 copies). Each one of these member is considered as potential candidate for the *S_H3* resistance gene.

PART II

Cloning *S_H3*-CNL candidate genes to CLR resistance

The analysis of *S_H3* loci in both sub genomes of *C. arabica* and in the genome of *C. canephora* species showed that the structure of the locus is conserved even if genome-specific insertion/deletion events have been evidenced (Chapter III). Using the same hypothesis that the structure of the *S_H3* locus is also conserved in the introgressed segment from *C. liberica* and that an orthologous copy exists for each identified member, a strategy based on cloning PCR amplified copies was implemented in order to isolate all the members of the *S_H3*-CNL family.

Material and Methods

Specific primers of each member present in sub genome E^a from *C. arabica* cv. IAPAR-59 were designed based on the flanking region (Table V.2). These primers were tested for amplification in *C. arabica* S.795 CLR resistant cultivar.

Table V. 2 Primers sequence designed to amplify candidate genes against to coffee leaf rust.

S_H3_R gene	LEFT PRIMER	RIGHT PRIMER
A1	AATATTCTGAACATGTGGTTTAGGCACTCT	TATATTCTATGATCGGCTGGTTCAAAACAT
A2	CCTTGATAAGAAACATGATGAAATACACGA	GTCCGAGTCATCAAGAAGAGTAAATAGGAA
A3	TCTGAAATTATCCCTTGTTATTTTGACTGC	GAAAATTTGAGCATTATTGAACACAGTGAG
B1	CAGCAAAGGAACATATGCCATTTGAAAG	GATCTGTTGAAGATTCCCTGCACCATAA
B2	TCTGTATGTTCCCCCAATTGAGCATTTA	GTCGACAAGTATGCCCTGAAAGAGTCTG

DNA amplification was essayed with three different polymerase enzymes: Expand^{TM} 20Kb^{plus} PCR System (Roche Applied Science, Mannheim Germany), High fidelity Platinum Taq (Invitrogen) and Gold Star (Eurogentec). When using the Expand^{TM} 20Kb^{plus} PCR System, PCR were performed as follows: one cycle of 1 min at 95°C, 10 cycles of 10 sec at 94°C, 45 sec at 50°C, 5 min at 68°C followed by 20 cycles of 10 sec at 94°C, 45 sec at 50°C, 7min at 68°C plus 10 sec per cycle, and final extension of 7 min at 68°C. For Platinum Taq (Invitrogen) and Gold Star (Eurogentec), PCR were

performed as follows: 4 min at 94°C, 35 cycles of 30 sec at 94°C, 30 sec at 55°C, 5 min at 68°C and final extension of 7 min at 68°C.

The amplicons were gel-stained using Crystal violet and the DNA bands were purified using a S. N. A. P™ purification column (Invitrogen Carlsbad, CA). The PCR products were cloned into pCR®-XL-TOPO® kit from Invitrogen and chemically competent cells (Invitrogen Carlsbad, CA) according to the manufacturer's protocol. Eight colonies were randomly selected for screening. PCR reactions of eight randomly chosen colonies were used to select bacterial clones containing the complete amplicon.

The colony PCR was performed as follows: 10 min at 94°C, followed by 30 cycles of 30 sec at 94°C, 30 sec at 55°C, 4 min at 72°C and final extension of 10 min at 72°C. The colonies that had been confirmed to contain the complete insert were transferred onto 5 ml Luria broth media with 50 µg/ml kanamycin and incubated at 37°C overnight. Plasmid DNA was isolated using Promega Wizard® *Plus* Minipreps DNA purification System (Promega Corporation, Madison, WI, USA) according to manufacturer instruction. Two independent PCRs and sequencing were carried out to verify the reproducibility of the information.

Plasmid DNA was sequenced at Genome Express (Grenoble, France) using M13-universal-forward and reverse primers and other five internal primers were designed using the Primer3 program (Whitehead Institute, USA) to allow whole gene sequence (Table V.3).

The cloned sequences were assembled using Staden package (Staden, 1996). The complete clone sequences were aligned using Bioedit v.7.0 (Hall, T.A. 1999) and compared with those of *C. arabica* IAPAR-59.

Table V. 3 Internal primers used for complete sequencing of S_H3_CNL members

INTERNAL PRIMERS	S_H3_CNL Members				
	A1	A2	A3	B1	B2
INT1	TGCGACGATTGTTGAAAGAC	TCCATCGTCCAAGATACAGC	CGAGGAATCAACAGCCTCAC	TCCATCGTCCAAGATACAGC	TCCATCGTCCAAGATACAGC
INT2	CCCAACAGTGCAATACGATG	TTTGTTGGGATGGAAGATGA	GAGGATGAAGGCTGGGAGTT	TTTGTTGGGATGGAAGATGA	TTTGTTGGGATGGAAGATGA
INT3	GTTGGCAAGTGTGGAAACCT	GCTGGGAGTTGCTTCAAAAG	TCGAATGTGGACAGCAGAAG	GCTGGGAGTTGCTTCAAAAG	GCTGGGAGTTGCTTCAAAAG
INT4	AAGTGGTGGCTGTGGATTTC	TCGAATGTGGACAGCAGAAG	CCGATTAAGGTGCCTGATGT	TCGAATGTGGACAGCAGAAG	TCGAATGTGGACAGCAGAAG
INT5	CCATAATCGGGGGAAGGTAT	GCCTTGGAGACACTTCCATC	CCAAAGAATTCATGGGATCG	GCCTTGGAGACACTTCCATC	GCCTTGGAGACACTTCCATC

Results and Discussion

All *S_H3*-CNL members specific PCRs on *C. arabica* accession S.795 as template produced amplicons sizing around 4000 bp. Among the clones obtained with specific primers for A1, B1 and B2

members, only the sequences of members belonging to the E^a subgenome were obtained. On the other hand, clones obtained with specific primers for A2 and A3 members contained two different types of sequence, from which, one was identical to the corresponding one on the E^a subgenome and the other was different from all known S_H3-CNL sequences. As expected, the sequences of the two new S_H3-CNL members corresponded to two of the five sequences obtained with the sequencing approach for the estimation of the number S_H3-CNL introgressed members (Table V.4). The sequence of the A3 member showed an interrupted open read frame (ORF) that prevent a full size translation and was considered as a pseudogene. On the contrary, the A2 member exhibits a conserved ORF and, consequently, represents of candidate for S_H3 resistance gene.

As aforementioned, we estimate that at least 4-5 of S_H3-CNL members have been introgressed into S.795. However, only two introgressed genes (corresponding to the A2 and A3 members) were amplified using this approach. The most probable explanation is that the member specific primers failed to anneal in the introgressed region. In fact, to ensure their specificity, the primers where designed on regions quite distant from the coding region. These regions are not expected to be under purifying selection and it is probable that they have undergone mutations that prevent the primer annealing.

Table V.4. Result of amplification of S_H3 members from *C. arabica* S.795 acession.

Gene	Type of sequence	OBS	Candidate gene
A1_E^a	1	Identical to E^a (non introgressed)	
A2_E^a	2	2 clones fully sequenced from liberica introgression	ORF conserved candidate
A3_E^a	2	2 clones fully sequenced from liberica introgression	ORF interrupted no candidate
B1_E^a	1	Identical to E^a (non introgressed)	
B_E^a	1	Identical to E^a (non introgressed)	

Conclusion

Only two genes from the 4-5 estimated introgressed S_H3-CNL members into *C. arabica* were cloned using the methodology employed. The A3 member is a pseudogene but the A2 member has conserved ORF and was considered a candidate gene.

PART III

Construction of expression vectors and functional gene validation

Two sequences were cloned using A2 specific primers. One of them was identical to E^a_A2 from IAPAR-59; the other was different from all the known *C. arabica* sequences and was therefore considered as introgressed from *C. liberica*. This S_H3-CNL copy has a conserved ORF and consequently represents a candidate gene for the S_H3 resistance. A complementation test was performed to verify the ability of this gene to induce S_H3 resistance. The *C. arabica* cultivar Caturra is susceptible to race II of *H. vastatrix* for which S_H3 gene is effective; for this reason the Caturra cultivar was used for coffee transformation experiments.

Material and Methods

a) Construction of expression vectors

We used the gateway technology to obtain expression clones containing the candidate gene (CG) A2 that was cloned from S.795 cultivar. The gateway system is considered as a universal cloning method that takes advantage of the site-specific recombination properties of bacteriophage lambda to provide rapid and highly efficient way to move the gene of interest into multiple vector system (Curtis and Grossniklaus, 2003).

As the first step into directional cloning, we amplify the CG adding a TAG (CACC) in the 5' primer and the fragment was cloned into pENTR/D-TOPO (Invitrogen). Two different reverse primers were designed with or without stop codon. The amplification of the CG gene without stop codon was generated to allow fusion with *GFP* gene. Once flanked by *att*L recombination site, the sequence was recombined with *att*R sites using LR clonase mix (enzymes from lambda bacteriophage - Integrase (Int), Excisionase (Xis) and the host integration factor (IHF) from *E. coli* producing the expression clone (Figure V.3).

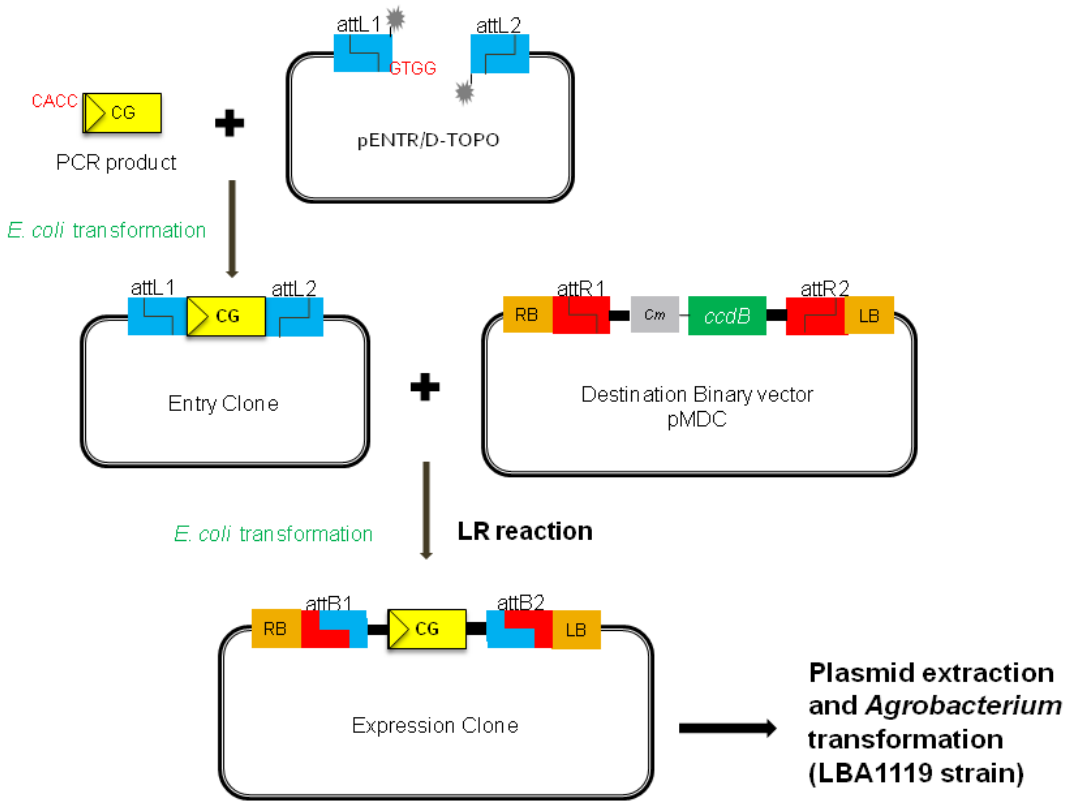

Figure V. 3 Construction of binary vectors used for plant transformation carrying out candidate R genes against CLR.

The vectors of pMDC series were used as expression clones (Curtis and Grossniklaus, 2003). Theses vectors have double 35S promoter and a gene for hygromycin selection *in planta*. The pMDC series of binary vectors are freely available for non commercial research (Curtis and Grossniklaus, 2003). Three different vectors were used. The pMDC43 and pMDC83 allow the fusion of the interest gene with Green fluorescent protein (*gfp6)* gene at C or N-terminal and are used to localize the interest protein, respectively. The pMDC32 vector has no *gfp6* fusion (Figure V.4).

The pENTR clone containing the CG was recombined with the pMDC vectors (*i.e* pMDC32, pMDC43, pMDC83) using the LR clonase enzyme. Since both plasmid (pENTR and pMDC vector) have the same resistance to kanamycin antibiotic, previously to the LR reaction, the pENTR carrying out the CG was digested with *Mlu* I enzyme that eliminate the kanamycin gene.

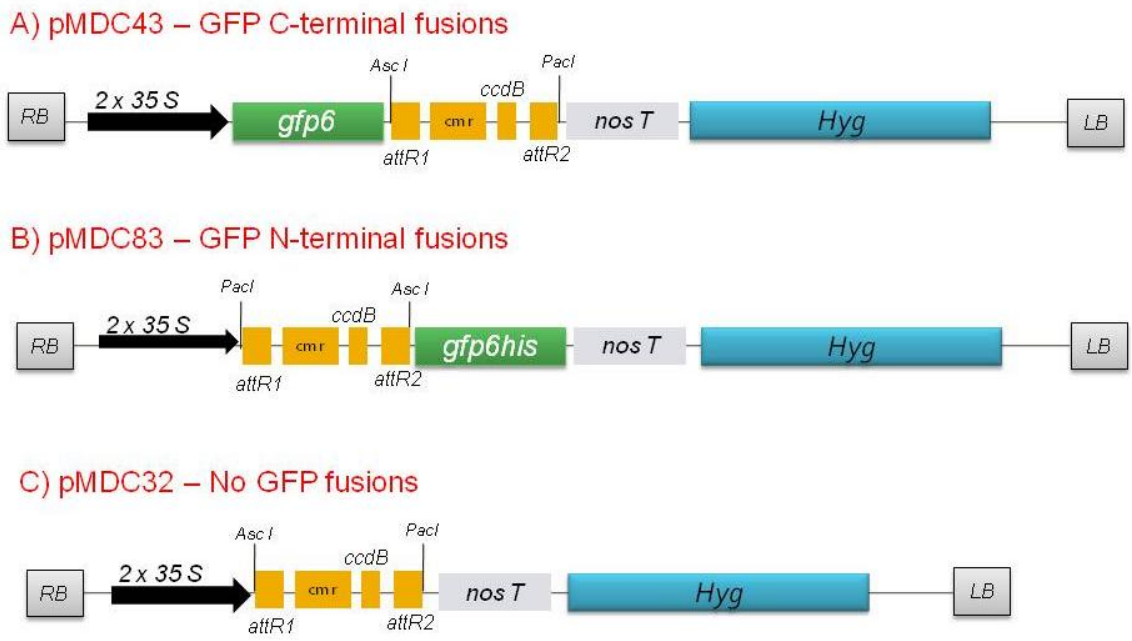

Figure V. 4 Expression cassettes used to clone candidates genes.

We used the vectors of pMDC series as expression clone. These vector has double promoter 35S and a gene for hygromycin selection in planta. The pMDC series of binary vectors is freely available for non commercial research (Curtis and Grossniklaus, 2003). Three different vectors were used. The pMDC43 and pMDC83 allow the fusion of the interest gene with Green fluorescent protein (gfp6) gene at C or N-terminal, respectively while the pMDC32 vector has no gfp6 fusion (Figure V.4) and are used to localize the interest protein.

The pENTR clone containing the CG was recombined with the pMDC vectors (i.e pMDC32, pMDC43, pMDC83) using the LR clonase enzyme. Since both plasmid (pENTR and pMDC vector) have resistance to kanamycin antibiotic, previously to the LR reaction, the pENTR carrying out the CG was digested with *Mlu* I enzyme that eliminate the kanamycin gene.

The LR reaction was achieved as follow:

150 ng of entry vector (pENTR)

150 ng of the pMDC vector

2 µl LR clonase mix enzymes

The reaction was incubated overnight at 25°C. To stop the reaction 1µl of K proteinase was add to the mix. The samples were incubated at 37°C during 1h. 1µl of reaction was added to chemically competent cell of *E. coli* (Top10). The cells were kept on ice during 30 min after incubated at 42°C for 30 s. 250 µl of SOC medium was added to each tube. The tubes were incubated at 37°C during 1h. 100 µl of bacterial culture was spread onto LB solid medium containing 50µl/mg kanamycin. Colonies that grew up under selective medium were analyzed by PCR and restriction. Positive clones were completely sequenced. After confirmation of CG integrity the expression clones were inserted into *Agrobacterium tumefaciens* strain LBA1119.

Genetic transformation coffee

We used the transformation methodology established in Chapter IV for CG validation in coffee plants (for details see Chapter IV). Once the embryogenic calluses are obtained, they can be sub-cultured each 3 weeks on the maintenance medium (MS medium containing 4.5µM 2,4D and 12 µM 6-BA, Etienne, 2005) until 12 months. Alternatively embryogenic calli competent for genetic transformation can be cryopreserved and used afterwards for transformation experiments. The 3 expression vectors were used to transform embryogenic cells of C. arabica cv. Caturra. Two different agrobacteria suspensions (0.6-0.8 OD_{600}) were grown up with each one of the expression clone. Each suspension was used to inoculate two jars containing coffee embryogenic calluses that have proliferated 4 months on the maintenance medium. From each jar 4 plate dishes containing 20 calluses approx. 3 mm diameter were made. A total of 320 calluses were inoculated for each expression vector. The negative control for transformation consisted of a jar inoculated with an A. tumefaciens LBA1119 without expression vector (Figure V.5). An illustrated general view of the transformation procedure is shown in Figure 6 V.

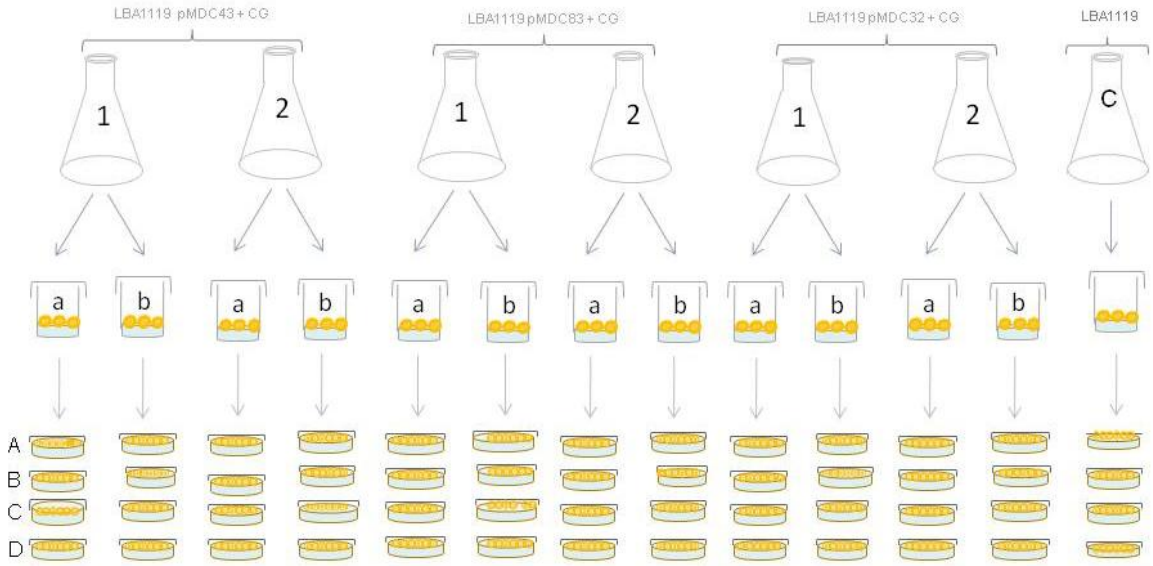

Figure V. 5 Schema representing the coffee transformation experiment for CG functional validation. Two *A. tumefaciens* suspensions (1 and 2) each carrying out the same expression clones were used inoculate to two jars (a and b) containing the embryogenic callus coffee. From each jar 4 plate dishes containing 20 calluses were made (A to D). One jar was inoculated with *A. tumefaciens* LBA1119 suspension without expression vector and used as negative control.

Analysis of transgenic coffee plants

Hygromicin and or GFP detection by PCR

Genomic DNA was isolated from leaves of putative transgenic and non transgenic nursery plants. The leaves were frozen in liquid nitrogen and DNA extraction was carried out with a CTAB procedure (Doyle and Doyle 1990) with modified extraction buffer (2% CTAB, 1.4mM NaCl, 100mM Tris HCl, 20mM EDTA at pH 0.8). Primers for amplification of a fragment HPTII gene: 5'-GCCTGAACTCACCGCGACGTC-3' and 5'-GCACTGACGGTGTCGTCCAT-3' (fragment size 506 bp) and for amplification of GFP6 gene: 5'-ATGAGTAAAGGAGAAGAACT-3' and 5'-TTAAAGCTCATCATGTTTGT-3' (fragment size 1000 bp)
PCR was carried out in a 25µl volume containing 50ng of genomic DNA, 1U Taq DNA polymerase, 1X buffer (Promega, Madison USA), 0.2 mM each dNTP, 2mM $MgCl_2$, 0.2 µM of each primer. Amplification was performed in thermal cycler GeneAmp® PCR system 9700, Applied Biosystem) in the following reaction: 1 cycle of 2min 95°C, followed by 30 cycles 1 min at 95°C, 1 min 55°C, 1 min

72°C and 4 min 72°C of final extension. The reaction products were electrophoresed in 1% (w/v) agarose gels and visualized by staining with ethidium bromide.

GFP visualization

Leaves and roots of transgenic coffee plants were observed using a fluorescence stereomicroscope A Leica MZ FlouIII (optic 0.63 Zeiss) supplied with a DC 300F camera (Leica Microsystem, Wetzlar, Germany), band-pass excitation filter (480 nm BP 40) and long-pass emission filter (510 nm LP) was utilized for visualization of *GFP*.

Rust resistance bioassay using transgenic coffee plants

Plantlets were inoculated using the procedure previously described by Silva et al. (2002). Briefly, the urediospores of *Hemileia vastatrix* race II were spread with a camel hair brush over the lower surface of young coffee leaves. Distilled water was then kindly sprayed on to the inoculated surface and the plants were kept for 24 h in a dark moist chamber. The symptoms were evaluated 4 weeks after inoculation.

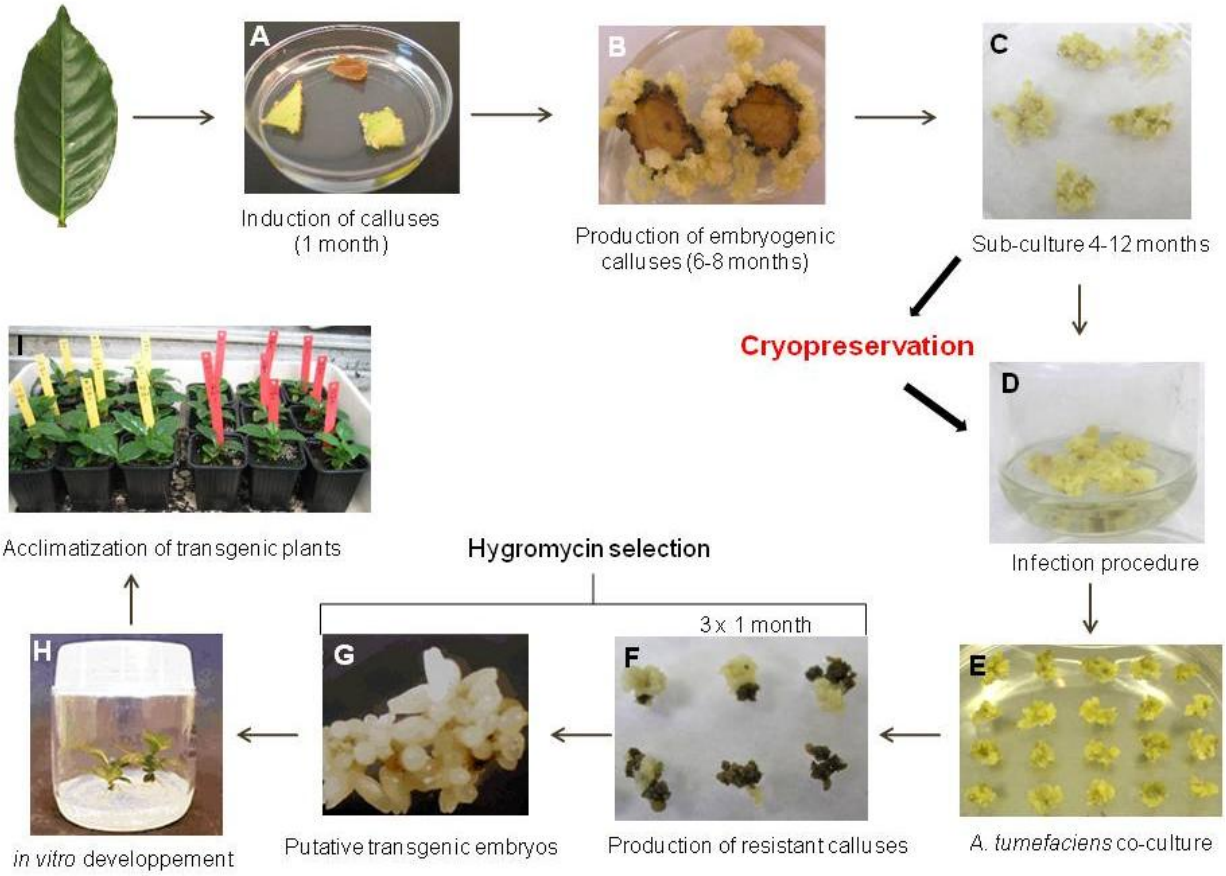

Figure V. 6 Overview of the transformation method used to produce transgenic coffee plants.

Results and discussion

The transformation of embryogenic callus was achieved following the protocol that we have established for coffee (for details see chapter 4). For each expression vectors constructed with A2 candidate gene, 320 calluses were co-cultivated. Four months after co-cultivation, 143, 210 and 251 hygromicin resistant calluses were produced for pMDC43+CG, pMDC83+CG and pMDC32+CG respectively, representing 44, 65 and 78% of transformation efficiency respectively.

A fragment of *HTPII* gene that confers resistance to hygromycin was amplified by PCR in all analyzed putative transgenic plants and a fragment of *GFP6* gene was amplified in those plants transformed with pMDC43 and 83. However, although the GFP gene was proved to be present in the transgenic plants, the subcellular localization of the fusion protein using a fluorescence stereomicroscope failed.

The presence of the GFP can lead to an improper folding of the fusion protein or hiding a localization signal that could be essential for a proper location. Moreover the vectors of pMDC series

used the *GFP6*, which is soluble into cytoplasm becoming less fluorescent. Woody species such as coffee trees are rich in autofluorescent compounds and sensitive detection of GFP in these species is sometimes difficult. For example similarly, no detection of *GFP6* was also observed in transgenic apple trees using fluorescence stereomicroscope (Hily and Liu, 2009).

A total of 30 transgenic coffee plants produced with each vector (pMDC43+CG or pMDC83+CG) and 60 with pMDC32+CG were successfully transferred to the greenhouse. Plants from twelve transformation events from pMDC32 containing the A2 candidate gene (2 plants for each event) were inoculated with the urediospores of *Hemileia vastatrix* race II. Two non transformed plants (hence, susceptible to coffee leaf rust rust) were also inoculated as positive controls. Thirty days after inoculation the yellow spots were observed in both transgenic and control plants; 10 days later the urediospores were produced (Figure V.7). This means that A2 member was not able to confer resistance to race two of *Hemileia vastatrix.*

Since at least 4-5 members were introgressed in the S.795 cultivar it is possible that other gene present into S_H3 locus be responsible for conferring the CLR resistance rather than A2 candidate, or that the combinatory expression of different members be required to confer resistance.

In some cases, more than one gene into a cluster of R genes is needs to confer resistance. One example is the locus Pikm that confer resistance to rice blast caused by *Magnaporthe oryzae* (Ashikawa et al., 2008). Two candidate NBS-LRR genes were found in this region and consider as candidates genes. Genetic complementation analysis of transgenic rice lines individually carrying each one of the genes do not conferred resistance to rice blast. However transgenic lines carrying both of these genes expressed blast resistance. Although the two genes contain all the conserved motifs necessary for to function independently as a resistance gene both are required to confer resistance to rice blast (Ashikawa et al., 2008). Similar results were observed by Lee et al. (2009).

Figure V. 7 Transgenic coffee plants carrying out the candidate genes A2 not conferred resistance to CLR. A: Apparition of yellow spots 30 days after inoculation with race II of *Hemileia vastatrix*. B: development of spores 45 days after inoculation.

As we were not able to clone all introgressed members and the only cloned member did not confer rust resistance, a new strategy to isolate the candidate genes was initiated. The new strategy consists in developing a pooled BAC library directly in the S.795 introgressed (Figure 8). This library has been constructed at INRA, Toulouse (http://cnrgv.toulouse.inra.fr/fr/services/non_gridded_bac_library). The previous determination of genetic and physical map of S_H3 locus from the IAPAR-59 cultivar will help the quick localization of the resistant locus making easy the isolation of all candidate genes in the S.795 cultivar. Currently, the S.795 pooled BAC library is been screened to localize the locus S_H3. The selected BACs will be completely sequenced and the candidate genes will be isolated. The same expression clones construction aforementioned will be employed to clone the CG. The transformation method that we developed in the present work represents a reliable tool for functional validation of CG in coffee as its high repeatability and efficiency was demonstrated.

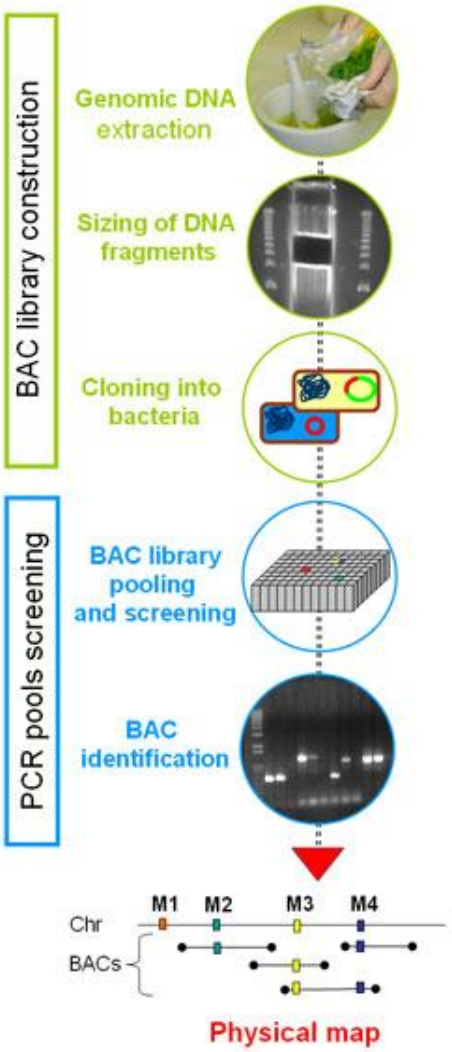

Figure V. 8 Strategy used for constructing a pooled BAC library of S.795 resistant cultivar to coffee leaf rust (http://cnrgv.toulouse.inra.fr/fr/services/non_gridded_bac_library).

CHAPTER VI

General Discussion and Perspectives

Coffee is one of the most important agricultural products in international trade. Coffee leaf rust (CLR), caused by the fungus *Hemileia vastatrix* is the main disease of cultivated *C. arabica* and it can substantially reduce the production of coffee beans if no disease management is employed. The major challenge of coffee breeders is to create coffee cultivars combining high yield, cup quality and durable resistance to pests and diseases. Although CLR can be controlled by chemicals, the most economical and ecologically friendly strategy to control the disease is the use of resistant cultivars. Hence, different breeding programs were developed with most of them using the Timor hybrid (introgressed by *C. canephora*) as the source of resistance. These programs resulted, since the eighties, to the selection and deployment on a large scale of resistant cultivars. However, examples of loss of resistance have been reported during recent years in different countries.

The cloning and the characterization of resistance genes can lead to a better understanding of R gene diversity and organization and of the molecular basis of plant-pathogen interactions. All these data can provide useful information to improve the breeding strategies to develop cultivars with durable resistance in field conditions. Towards this objective, this thesis presents the results of efforts focused on the S_H3 locus from coffee that confers resistance to the CLR.

1. Positional cloning approach

The resistance provided by S_H3 is dominant (Prakash et al., 2004). The S_H3 resistance factor was introgressed into *C. arabica* gene pool through a natural hybridization with *C. liberica*. As a first step to positional cloning of the S_H3 resistant factor, the genetic and physical mapping of S_H3 region was realised in *C. arabica* with the help of a BAC library from cultivar IAPAR-59 (Chapter II). Although this cultivar is resistant to coffee leaf rust, it lacks the *C. liberica* introgression and consequently the S_H3 resistant factor.

The complete sequence of the S_H3 locus (550 kb) in three coffee genomes (E^a and C^a) from *C. arabica* cv. IAPAR-59 and C^c genome from *C. canephora* accession HD 200-94 revealed the presence of a variable number of potential R genes (3-5, according to the genome considered) belonging to a family of CC-NBS-LRR (CNL) class of genes. Southern hybridization and sequence analysis of partial PCR-amplified S_H3-CNL members from a *C. liberica*-introgressed genotype (S.795)

indicated that while IAPAR-59 has 8 *S_H3*-CNL members, the resistant cultivar S.795 contains at least 9 or 10 *S_H3*-CNL members. A comparative analysis between the *S_H3* loci in the two cultivars demonstrated that the introgressed segment from the *C. liberica* genome has occurred into C^a sub-genome of *C. arabica* (Chapter II). Since five (5) *S_H3*-CNL members were localized in E^a sub genome of *C. arabica*, then at least 4 or 5 members were inferred to be present into the *C. liberica*-introgressed segment carrying the *S_H3* resistant factor in the cultivar S. 795 and therefore were considered as candidate genes to CLR.

The challenge to clone all the candidate genes (CG) for the *S_H3* resistant factor from cultivar S.795 was initially based on direct PCR-amplification of the *S_H3*-CNL members using specific primers targeting the flanking regions of the different members identified in *C. arabica*. This approach was partially successful since only two genes corresponding to introgression from *C. liberica* were cloned (A2 and A3 members). The A2 introgressed gene had a conserved ORF and appeared clearly as a good candidate gene that is involved in the *S_H3* resistant factor. On the contrary, the A3 sequence showed an early stop codon that prevents the complete translation of the gene which was consequently excluded from the candidate genes. As aforementioned, it was estimated that 4-5 copies have been introgressed into the cultivar S.795. However, only two introgressed copies were amplified using this approach, probably because the flanking regions are not conserved between *C. arabica* and *C. liberica* or the members detected in *C. arabica* are not present in the *C. liberica*-introgressed segment.

Since this approach was not efficient to identify all the CGs present in the the introgressed resistant genotype S.795, a non gridded BAC library from this cultivar was recently constructed (http://cnrgv.toulouse.inra.fr/en/services/non_gridded_bac_library). The screening of this library in collaboration with INRA-CNRV allowed the identification of a few BAC clones containing *S_H3*-CNL members (Figure VI.1). The sequencing of these BAC clones is now in progress and the sequences of the other candidate genes will soon be available. In order to individuate the gene(s) responsible for the resistance, a complementation test on a susceptible genotype will be performed with all the candidate genes.

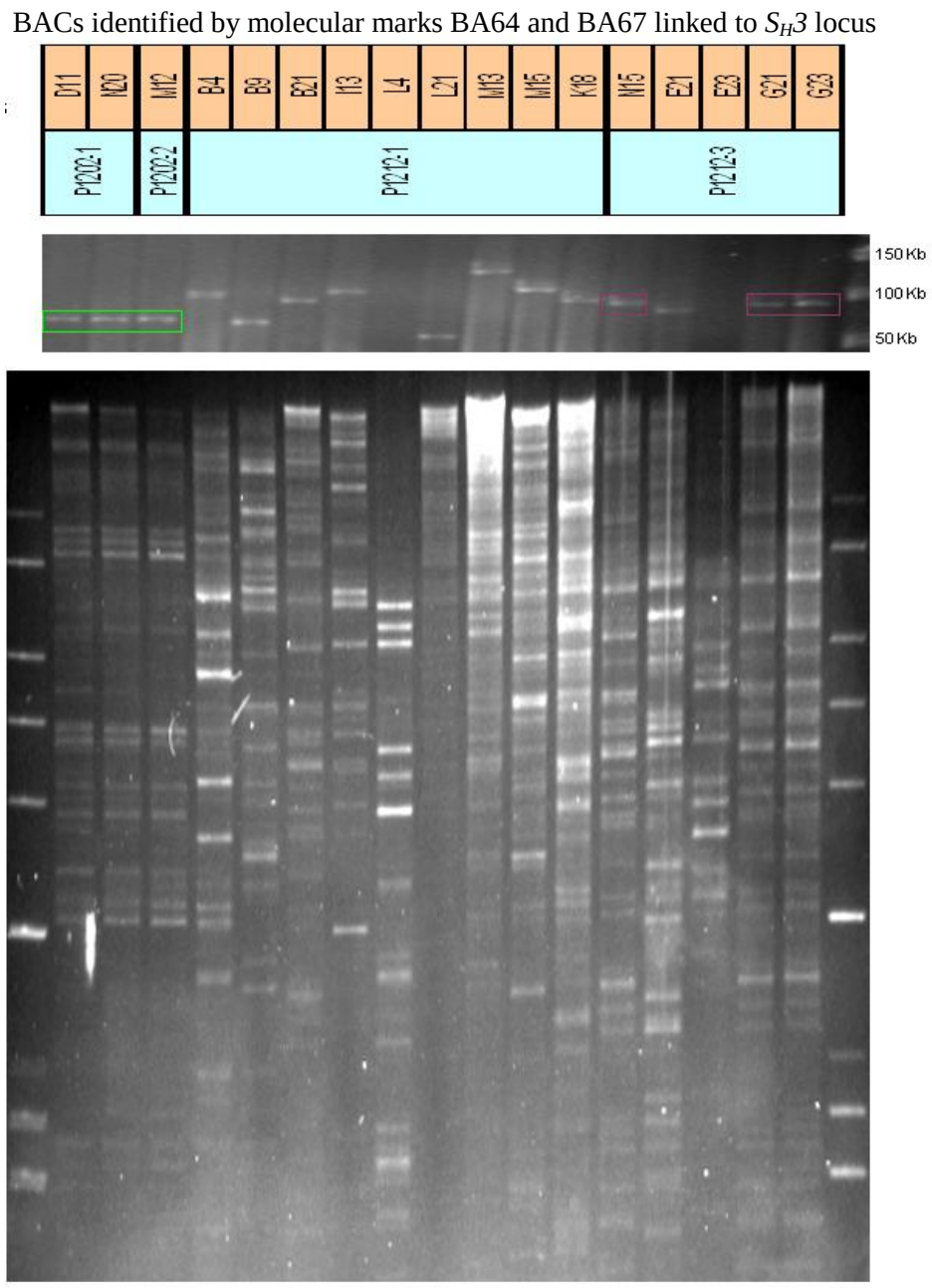

Figure VI. 1 Identification of BAC clones containing the S_H3 locus in the S.795 resistant cultivar of *C. arabica*. A. BAC clones containing the S_H3 locus; B. Pulse field of BACs digested with Not I enzyme; C. BACs fingerprint digested by *Eco* RI enzymes.

2. Development of tools for reliable functional gene validation

Coffee leaf rust is caused by the biotrophic fungus *Hemileia vastatrix*, a specific disease of coffee plants. In such a case of tight relation/co-evolution between host and pathogen, functional validation of candidate genes of resistance to coffee leaf rust cannot be conducted in model plants such as *Arabidopsis* or tobacco, for which, highly efficient genetic transformation procedures have been established. On the other hand, reliable transformation procedures have yet to be established in most of woody plants including coffee trees. Different reasons can explain this delay in developing efficient tools for the functional validation in woody plants. First, the global recalcitrant character to *in vitro* culture probably associated to i) the long biological cycles directly related to the long-term morphogenetic responses and ii) the high oxidative processes and elevated phenol contents generally found in perennial species. Secondly, the *in vitro* regeneration systems on which the genetic transformation protocols are based do not allow the plant regeneration from adult tissues for a lot of species. Therefore, for such species, transformed plants are derived from zygotic embryos or young seedlings, and consequently do not reproduce exactly the same genotype of the studied variety. Consequently, due to the difficulty to work on woody species most of researchers prefer to work on model plants where the available high throughput transformation technologies and related genomic tools allow rapid, reliable and quantitative generation of data necessary for a more fundamental and attractive research.

Even if different transformation technologies have been occasionally employed with perennial species (particle bombardment, protoplast electroporation), the currently most employed method is the use of the natural vector, *Agrobacterium tumefaciens*, put in contact with *in vitro* cultured explants that have the capability to regenerate whole plants using the somatic embryogenesis or adventitious regeneration pathways. Various coffee research groups mainly in Brazil (Embrapa), Colombia (Cenicafe), Japan (Ochanomizu University), Venezuela (University of Venezuela), India (Tissue Culture and Biotechnology Center of Coffee Board) and France (Nestlé and CIRAD-IRD) have used several methods, different explants and culture conditions to transform coffee tree. Although some success has been achieved, particularly through the production of transgenic plants expressing some agronomically interesting genes such as resistance to insect and silencing genes of caffeine biosynthesis, coffee transformation efficiency is still very low and the production of transgenic plants has not been systematically obtained (Leroy et al., 2000; Ogita et al., 2004; Ribas et al., 2005, Albuberque et al., 2009). At the beginning of this thesis work, no reliable protocol for coffee transformation existed for *C. arabica*. In fact, no extended analysis had been achieved about the physiological status of target cells and this is known to be a crucial step for successful plant

transformation. The main bottleneck to achieve a reliable transformation in *C. arabica* is the difficulty to obtain embryogenic tissues and to establish the optimal conditions for their proliferation. Another problem is to obtain target materials in enough quantities for further transformation experiments. In the present work it was demonstrated that the establishment of such proliferation conditions for embryogenic callus was a necessary but not sufficient step to ensure efficient transformation. Indeed, it was demonstrated that the culture conditions of the embryogenic callus cultures previous to co-culturing with the agrobacteria, and hence the physiological status of embryogenic cells, strongly influenced the success of genetic transformation.

The process of establishing coffee embryogenic cultures led to the development of three morphologically different callus phenotypes with highly contrasting potentials for genetic transformation. The yellow type selected within the optimal 6-14 month proliferation period appeared to be an ideal material for transformation purposes (up to 95% transformation efficiency). This exceptionally high successful transformation rate could be due to the enrichment in transformation competent target cells occurring during the established selecting process based on the two parameters: callus phenotype and age. The highly competent cultures are effectively constituted of only pro-*embryogenic masses* (PEM). Similarly, PEM cells have been previously identified as the target tissues for *A. tumefaciens* transformation of *Arabidopsis thaliana* (Sangwan et al., 1992) and *Vitis rotundifolia* (Dhekney et al., 2008).

Although the methodology established in this study for coffee transformation is highly efficient and reliable, the process is still long to be used in high throughput functional genomics. On average, 27 months are required to obtain transgenic coffee plants starting from the initial leaf explant. The best alternative to reduce this time is probably the use of cryopreserved tissues. Once established the best conditions for embryogenic callus proliferation and transformation, the callus can be cryo-preserved for long-term storage as germplasm to be used in future transformation experiments avoiding maintenance, ageing of tissues and disappearance of competent cells (Jain, 2006). The use of cryo-preserved competent coffee cells decreases to 12 months the process duration required to obtain transgenic coffee plants (see Figure VI. 1). Preliminary results of coffee transformation using cryopreserved embryogenic tissues were very encouraging (Fourty, 2009). Interestingly, the performance of cryopreserved tissues was higher than non-cryopreserved tissues. Up to 70% of stable GFP expression was observed in cryopreserved tissues against 40% in non-cryopreserved. The positive effect of cryopreservation on the success of the transformation could be due to the resulting process of “cellular selection” of PEMs occurring during the cryopreservation procedure. Is is well known that embryogenic cells proved to be more resistant to different stresses including cryopreservation.

PEMs and embryogenic cells exhibit characteristics of metabolically very active cells with prominent central nucleus, visible nucleolus and small vacuole, along with large starch, protein and lipid reserves. These features make them probably resistant to most stresses including cryopreservation and consequently more competent to a range of biological processes like somatic embryogenesis and genetic transformation. These reserves are important to restart the cellular activity after bearing a stress such as *Agrobacterium* infection for example. A bank of cryopreserved competent coffee tissues for transformation has been established in our laboratory and actually allows the routine functional validation of coffee candidate genes. In theory, any candidate gene could be functionally validated and since the developed transformation method is supported by a very efficient somatic embryogenesis procedure, large quantities of fully transformed plants can be regenerated.

It is well known that the transformation efficiency generally decreases in line with the length of genes to insert in plant genomes via *Agrobacterium*-T DNA. Although the tested CLR candidate gene has approximately 4000 bases and hence corresponds to a long gene, very high transformation efficiency was achieved. The selection of coffee transgenic tissues based on the resistance to Hygromicin was demonstrated to be highly efficient since no escape was detected among all the analyzed transformation events. This result is in agreement with those recently published on coffee tree by Mishra et al. (2006). Furthermore, fifty percent of the plants were proven to have integrated only one transgene insertion. Variations in the level of expression of foreign genes have been partly attributed to differences in the number of transgene insertions (Bhat, 2002). Hence, it is more desirable to produce plants containing only one insertion copy of the candidate gene in view of functional genomics purposes to suppress the variations associated to the number of insertions.

In conclusion, the transformation methodology that was developed here fulfills the different requirements to be used for functional genomics and could in future become the reference protocol for functional validation of agronomically interesting genes in coffee. Several candidate genes are already available from EST collections (Vieira et al., 2006; Fernandez et al., 2004), microarray projects (Privat et al, in press) and from the present work. The coffee genome sequencing and annotation of *C. canephora* Pierre species (Argout et al., 2010) is now in progress and will rapidly generate thousands of candidate genes that must have their functions validated.

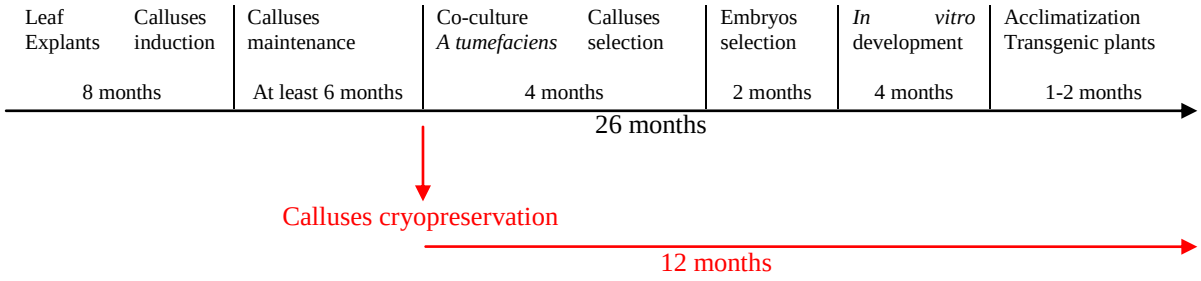

Figure VI. 2 Compared time durations for producing transgenic coffee plants using leaf explants versus cryopreserved calluses

3. Diversity and evolution of R genes in *Coffea*

The genomic sequence analysis of the S_H3 locus in three coffee species allowed characterizing the evolutionary features of the S_H3-CNL family. It was demonstrated that: (i) this family evolved by a birth-death model, (ii) gene conversion events (intra and inter-genomes) were frequent among the S_H3-CNL members; (iii) diversifying selection appears to act in the LRR regions (Chapter, III). It has been previously suggested that gene conversion plays a major role in evolution of NBS-LRR genes for at least two perennial species: grapevine and poplar (Yang et al., 2008). The characterization of S_H3-CNL genes in coffee seems to confirm the hypothesis that gene conversion is one of the most important mechanisms to generate gene diversity in perennial plants.

In addition, S_H3-CNL genes have sequence similarity with several well characterized resistance genes. These features are consistent with a role of the S_H3-CNL family in the recognition of pathogen aggressions and this makes it a very good candidate to CLR resistance, as well as for other biotic aggressors, such as nematodes (*Meloidogyne sp, Pratylenchus spp)* and fungal diseases (*Colletotrichum kahawae, Cercospora coffeicola, Fusarium stilboides, Hemileia coffeicola, Fusarium xylarioides*).

In fact, it has been demonstrated that R genes within a cluster can determine resistance to different pathogens (Michelmore & Meyers, 1998). For example: the locus *Rpg1* from soybean confers resistance against to a diverse kind of pathogens including: *Pseudomonas syringae, Phytophthora soja,* soybean mosaic virus and peanut mottle virus (Asfield et al., 2004). Two highly similar genes located a distance of 115 kb apart on the potato chromosome 12 confer resistance to two unrelated pathogens: potato cyst nematode and potato virus X (van der Vossen et al., 2000). *RPP8* and *HRT* from *Arabidopsis* are paralogous genes that encode resistance to *Peronospora parasitica* and turnip crinkle virus respectively (Cooleya et al., 2000). Similarly, the *Mi* locus from tomato encodes

resistance to a nematode and an aphid species (Rossi et al., 1998 and Vos, P. et al., 1998; Seah et al., 2007).

The characterization of the S_H3 locus can also be used in syntenic approaches to isolate resistance gene analogs or potential novel resistant alleles in the ortholog regions of closely related species. The exploitation of the available genetic diversity for resistance genes in cultivated and wild species of coffee could be very useful in the frame of breeding strategies aiming at the development of cultivars exhibiting durable resistances.

Furthermore, the whole sequencing and annotation of the coffee genome (*C. canephora* Pierre) that are now in progress (Argout et al., 2010) will provide the opportunity to study the number, distribution, genomic organization and the diversity of R genes in the *Coffea* genome. In particular, it will be possible to confirm the importance of gene conversion as general mechanisms to generate gene diversity in R genes.

4. Interactions between S_H3-gene and *H. vastatrix* effectors

Plant resistant proteins recognize a pathogen invasion interacting with their effectors. Any effector recognized by a R protein is qualified as an avirulence gene (Avr) (Dangl and McDowell, 2006). Pathogen virulence effectors that trigger indirect recognition are often found as presence–absence alleles across a pathogen species (Dangl and Jones, 2006) and can only escape detection by becoming non-functional. On the other hand, effectors proteins that are recognized by a direct physical interaction with the R protein, can avoid detection by altering binding sites while they maintain their function (Catanzariti et al., 2010). Pathogens with narrow host ranges and an inability to survive apart from their hosts (obligate biotrophs like rust) may have undergone evolution of virulence effectors with subtle effects that do not result in altered or modified host machinery (Dodds et al., 2006). The determination of molecular basis between R gene-Avr protein interactions could reflect the co-evolution model of S_H3 and effectors from *H. vastratrix.*

The direct interaction between R and Avr genes could be conducted using yeast two-hybrid assay. In this method, GAL4 DNA binding domain (BD) and transcriptional activation domain (AD) are merged with R and Avr proteins respectively. Coexpression of these protein fusions activate expression of the HIS3 and lacZ reporter genes in transformed yeast cells, indicating that these corresponding R and Avr proteins can form physical interactions in yeast. This method has been used to determine direct interaction in rust-flax system between AvrL567 effectors and the corresponding L proteins (Dodds *et al.*, 2006) and between M and AvrM (Catanzariti *et al.*, 2010).

The whole sequencing of *H. vastatrix* genome is now in progress and will soon open new experimental perspectives. Random sequencing of coffee rust genome using 454 technology obtained 16Mb of data in Colombia (Escobar et al., 2010). A total of 5,629 sequences were functionally annotated. From this, around 100 annotated sequences seem to be related to virulence as revealed by similarity with RXRL effectors with other plant pathogens and related proteins (Escobar et al., 2010 ASIC). Moreover, an EST database realized at three differentiation/infection stages (germinated uredospores, apressoria and haustoria) from an isolate of race XIV (containing the virulence genes v2, 3, 4, 5) has been created (Talinhas et al., 2010).

The isolation of race-specific Avr and R genes in other plant species has generated new opportunities for studying the mechanisms of plant-pathogen interaction. For example, AVR3a (a RXLR cytoplasmic effector) from *Phytophthora infestans* and each gene belonging to the R3 locus from potato (which possess 4 paralogs from NB-LRR class: R3-3, R3-1, R3-3, R3-4) were co-expressed by agro-infiltration in *N. benthamiana*. The results indicated that AVR3a is specifically recognized by R3a among the examined NBS-LRR genes of the R3 locus (Bos et al., 2006).

Similar experiments could be envisaged in the future to study gene pair interaction between S_H genes from coffee and effectors proteins from *H. vastatrix.* The isolation of R genes from coffee together with the identification of effectors genes from *H. vastatrix* involved in pathogenicity will provide a better understanding of the molecular basis of coffee-rust interaction.

5. Durable resistance to Coffee Leaf Rust

Durable resistance refers to resistance that remains effective during its prolonged and widespread use in environments favorable to the pathogen or disease spread (Johnson, 1981).

Biotrophic pathogens such as *H. vastatrix* are usually able to overcome host resistance by deletion or mutation of *Avr* genes leading to failure of R gene-mediated recognition and subsequent hypersensitive response (HR) and other responses (van der Vossen, 2005).

Resistance to diseases provided by single dominant R genes is sometimes ephemeral due to evolution of new virulent races. The most durable resistance genes are those that require multiple mutations by the pathogen for virulence, with mutations causing the highest fitness penalty (Palloix et al., 2009). The gene pyramiding approach (*i.e.* simultaneous introduction of several R genes targeting different races of the same pathogen into one elite breeding line) should provide a broader and more durable resistance since mutational events in multiples *Avr* genes will be required to produce new virulent pathogens (van der Vossen, 2005). The gene pyramiding assisted by MAS (Molecular assisted selection) has been used in many species to improve disease resistance.

The best DNA markers are those derived from sequences of the R genes themselves or their adjacent flanking regions. Thus, R gene sequences can be used to create precise 'within the gene' (WTG) molecular markers, without the need for pathogen testing during introgression breeding. Markers derived from R gene sequences therefore allow each gene to be traced accurately through each breeding generation without the need to carry out difficult and laborious phenotypic screenings (Hammond-Kosack and Kanurya 2007).

During the last few years, several rust resistance factors have been genetically analyzed, and associated molecular markers were reported. In contrast to the present work on the *S_H3* resistance factor inherited from *C. liberica*, the other works were focused on rust resistance factors introgressed into *C. arabica* from *C. canephora* through the Timor hybrid. The developed markers concerned a partial rust resistance (Herrera et al. 2009; Romero et al. 2010) and a complete resistance gene (de Brito et al. 2010). These introgressed regions carry resistance genes that are related the resistance factors known as *S_H6* to *S_H9*. However, the relationships are not yet clearly established (Diola et al., 2010) and preliminary data showed that the S_H3 region is different from those identified in the Timor hybrid.

All together, the characterization of the *S_H3* gene sequence as well as those from others *S_H* genes could facilitate the traceability of the introgression of these genes into elite cultivars using the pyramiding approach. Consequently the pyramiding of *S_H3* and other *S_H* resistant genes into elite coffee genotypes should provide a broader spectrum and more durable disease resistance to coffee leaf rust.

Similarly, different alleles of one gene, or the same alleles (allele-dosage) (Tan et al., 2010) could also be used in pyramiding approach. It has been demonstrated that pyramiding of different alleles of *Pm3* locus from wheat permitted a combined and thus broader resistance compared with those conferred by the parental alleles (Brunner et al., 2010).

Such pyramiding strategy could be facilitated by the development of a new and promissing type of variety based on F1 hybrids (Bertrand et al. 2005). Such *C. arabica* intraspecific F1 hybrids were selected in Central America from 1991 to 2006 and are currently propagated on a large scale through somatic embryogenesis (Etienne 2005; Menéndez-Yuffa et al., 2010; Etienne et al., 2010). In addition to high productivity and strong homeostasis associated to heterosis, this type of variety offers the possibility of developing new rust resistance varieties in less time (around 10 years). In particular, durable resistance could be achieved by the combination in F1 hybrids of complementary S_H genes inherited from both parents and therefore making it more difficult for *H. vastatrix* to break the resistance.

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Characterization of a locus of resistance to coffee leaf rust (*Hemileia vastratrix*) in coffee trees: genomic organization, diversity and development of tools for functional gene validation

Coffee leaf rust (CLR) caused by the biotrophic fungus *Hemileia vastatrix* (Berk & Br.) is the most devastating disease of Arabica coffee (*Coffea arabica*). Resistance to CLR in its allotetraploid species appears so far conditioned primarily by 9 major resistance factors (S_H1-S_H9) either singly or in combination. Arabica varieties harboring the S_H3 resistant factor introgressed from a wild coffee species (*C. liberica*) have demonstrated agronomical acceptable durable resistance in field conditions. The main objective of this work was to study the genomic organization, evolution and diversity of S_H3 locus in coffee and in parallel to develop tools for functional gene validation of resistance candidate genes to CLR. As the first step, a physical map spanning the S_H3 region in *C. arabica* was constructed and the position of the resistance locus was delimited within an interval of 550 kb. The region was completely sequenced in three coffee genomes, E^a and C^a subgenomes from *C. arabica* and C^c genome from *C. canephora*. Sequence analysis revealed the presence of a variable number of members CC subclass of NBS-LRR genes thatappeared to be exclusively present at this locus in *C. arabica*. Comparative genomic analysis indicated that i) the origin of most of the S_H3-CNL copies predates the divergence between *Coffea* species ii) the ancestral S_H3-CNL copy was inserted in the S_H3 locus after the divergence between Solanales and Rubiales lineages. Furthermore, duplications, deletions, gene conversion and positive selection appeared as the main forces that drive S_H3-CNL evolution. Different approaches have been undertaken to clone candidate genes to CLR. In parallel, a highly efficient and reliable *Agrobacterium tumefaciens*-mediated protocol was established for coffee. Transgenic plants containing one of the candidate gene were successful produced and acclimated into the greenhouse. The preliminary bioassay against *H. vastatrix* was achieved. The setting-up of efficient protocols for cloning resistance genes and for genetic transformation paves the way for routine functional genomics in coffee. The better knowledge on CLR resistance gene diversity would allow optimization of breeding schemes for durable resistance to this major coffee disease.

Key-words: Coffee, resistance gene, molecular evolution, molecular evolution, rust, genetic transformation

Charactérisation d'un locus de résistance à la rouille orangée (*Hemileia vastatrix*) chez le caféier (*Coffea arabica*): organisation génomique, diversité et développement des outils pour la validation fonctionnelle

La rouille orangée du caféier causée par le champignon biotrophe *Hemileia vastatrix* (Berk & Br.) est la maladie la plus dévastatrice du caféier (*Coffea arabica* L.). La résistance à la rouille dans l'espèce allotétraploïde *C. arabica* semble jusqu'à présent conditionnée principalement par 9 facteurs de résistance majeurs (S_H1-S_H9), seuls ou en combinaison. Les variétés d'Arabica contenant le facteur de résistance S_H3 introgressé d'une espèce de caféier sauvage (*C. liberica*) ont démontré une résistance durable au champ. L'objectif principal de ce travail était d'étudier l'organisation génomique, l'évolution et la diversité du locus S_H3 chez le caféier et parallèlement de développer des outils permettant la validation fonctionnelle des gènes candidats pour la résistance à la rouille. Comme Une carte physique couvrant la région S_H3 a tout d'abord été construite chez *C. arabica* et la position du locus de résistance a été délimitée dans un intervalle de 550 kb. La région a été séquencée chez trois génomes de caféier, E^a et C^a sous-génomes de *C. arabica* et C^c génome de *C. canephora*. L'analyse des séquences a révélé la présence d'un nombre variable de membres de la sous-classe CC-NBS-LRR ; ces gènes semblant être exclusivement présents à ce locus chez *C. arabica*. L'analyse génomique comparative indique que i) l'origine de la plupart des copies S_H3-CNL est antérieure à la divergence entre les espèces de *Coffea*, ii) la copie ancestrale S_H3-CNL a été insérée dans le locus S_H3 après la divergence entre les Solanales et Rubiales. En outre, les mécanismes de duplication, délétion, conversion génique et de sélection positive semblent être les principales forces qui déterminent l'évolution des membres de la famille S_H3-CNL. Différentes approches ont été entreprises pour le clonage des gènes candidats de résistance à la rouille. En parallèle, un protocole très efficace de transformation par *Agrobacterium tumefaciens* a été développé chez le caféier. Des plantes transgéniques contenant l'un des gènes R candidats ont été produites et acclimatées. Le bio-essai pour évaluer la résistance à *H. vastatrix* a été mis au point. La mise en place de protocoles efficaces pour le clonage des gènes de résistance et la transformation génétique ouvre la voie à la génomique fonctionnelle en routine chez le caféier. L'amélioration des connaissances sur la diversité des gènes de résistance à la rouille permettra l'optimisation des schémas de sélection pour la résistance durable à cette maladie majeure du caféier.

Mot-clés: Caféier, gènes de résistance, évolution moléculaire, rouille, transformation génétique.