UNIVERSITE CATHOLIQUE DE LOUVAIN
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Polymyxa betae - *Beta vulgaris*: understanding the molecular interactions through transcriptome and plant defense analysis

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John Wiley and Sons granted the permission for inserting picture from *Molecular Plant Pathology* in this thesis.

CONTENTS

ABBREVIATIONS

AIS	Automatic immersion system
ASM	Acybenzolar-s-methyl
Avr	Avirulence
BLAST	Basic local alignment search tool
BNYVV	*Beet necrotic yellow vein virus*
bp	base pairs
BSBMV	*Beet soil-borne mosaic virus*
BSBV	*Beet soil-borne virus*
BTH	Benzodiathiazole
BVQ	*Beet virus Q*
CAP	Common Agricultural Policy
cDNA	complementary DNA
CGB	Confédération générale des planteurs de betteraves
cLPs	cyclic lipopeptides

CLS	Cercospora leaf spot
CP	Coat protein
CP-RT	Coat protein, readthrough domain
Cq	Quantification cycle
dai	days after inoculation
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
dpi	days post-inoculation
ESTs	Expressed sequence tags
ET	Ethylene
ETI	Effector triggered immunity
ETS	Effector triggered susceptibility
EU	European Union
GFG	Gene for gene
GO	Gene ontology
GST	Glutathione-S-transferase
HKG	Housekeeping gene
HR	Hypersensitive response
ISR	Induced systemic resistance
ITB	Institut technique de la betterave
ITS	Internal transcribed spacers
JA	Jasmonic acid
LAR	Local acquired resistance

LDM	Long distance movement
LRR	Leucine rich repeat
MAMPs	Microbial associated molecular patterns
MIPS	Munich information center for protein sequences
MIQE	Minimum information for publication of quantitative real-time PCR experiments
mRNA	messenger RNA
MTR	methyltransferase
NCBI	National Center for Biotechnology Information
PAL	Phenylalanine ammonia lyase
PAMPs	Pathogen associated molecular patterns
Pb+	*P. betae* inoculated
Pb+	*Polymyxa betae* inoculated
Pb-	non-inoculated
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGPF	Plant growth promoting fungi
PGPR	Plant growth promoting rhizobacteria
PR	Pathogenesis-related
PRLs	Pathogenesis-related like
PRR	Pattern recognition receptor
PTGS	Post-transcriptional gene silencing
PTI	PAMPs triggered immunity

qRT-PCR	Real-time quantitative reverse transcription PCR
RACE	Rapid amplification of cDNA ends
RAPD-PCR	Random amplification of polymorphic DNA PCR
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RNAi	RNA interference
ROS	Reactive oxygen species
rRNA	ribosomal RNA
SA	Salicylic acid
SAGE	Serial analysis of gene expression
SAR	Systemic acquired resistance
siRNA	small interfering RNA
SSH	Suppression subtractive hybridization
TAIR	The *Arabidopsis* information resource
TGB	Triple gene block
TIGR	The Institute for Genomic Research

GENERAL INTRODUCTION

Problem statement

Sugar beet is one of the most important crops in the world. It is the ninth most produced commodity in the world and the second in Europe, which provides more than 70 % of global production. As other major crops, sugar beet is susceptible to a broad range of parasites, from insects to viruses, causing significant yield losses.

In the middle of 1960's, two papers concerning one of the present most severe diseases of sugar beet were published. The first described a new parasite of the sugar beet: *Polymyxa betae* (Keskin, 1964). The second paper related the discovery, in 1952, of a new disease of sugar beet called rhizomania (Canova, 1966). The causal agent of this disease was identified as a virus in 1971 and named *Beet necrotic yellow vein virus* (BNYVV) (Tamada *et al.*, 1971). This virus could reduce the yield up to 80%. A few years after the identification of the virus, the vectoring abilities of *P. betae* for the BNYVV were evidenced (Fujisawa & Sugimoto, 1977). The control of this disease is difficult because no phytopharmaceuticals are available against viruses. Moreover, the virus and the vector can survive for years in the soil. These characteristics of this pathosystem led to the use of resistant cultivars against the virus as the only means to control the disease.

Nevertheless, during the last decade, resistance breaking isolates of

BNYVV were identified (Acosta-Leal *et al.*, 2010; Pferdmenges *et al.*, 2009). Given the limited number of tolerance genes against the virus and the unavailability of specific phytopharmaceuticals or chemicals, research on *P. betae* experiences now a resurgence of interest, notably by seed producers. In this view, the understanding of the molecular interactions between the protist and sugar beet during the infection as well as the highlighting of involved sugar beet metabolic ways during this process could provide basis for new strategies of disease control.

The principal objectives of this thesis are to highlight genes as well as metabolic ways involved in the interaction *P. betae*-sugar beet.

Thesis outline

This thesis begins with a literature review presenting the state of the art of the different fields tackled in this thesis: *Beta vulgaris*, its cropping system and the rhizomania pathosystem including the BNYVV and its vector *P. betae*. Finally, this review deals with the study of the interactions between protists close to *P. betae* and their respective hosts. The main techniques and models used in this thesis are described: *Arabidopsis thaliana* and hairy roots, as models to investigate plant-parasite interactions are exponed as well as the differential transcripts analysis concepts and subtractive suppressive hybridization. Finally, plant defenses, important in the interaction plant-parasite, are described.

In the chapter 2, we present the development of two models to study sugar beet-*P. betae* interactions. The first aimed to identify *A. thaliana* ecotypes compatibles with *P. betae*. Secondly, we developed an *in vitro* dual culture of *P. betae* on sugar beet hairy roots, allowing to produce a pure inoculum of *P. betae*, free of other microorganisms.

The chapter 3 presents the results of a transcriptomic analysis performed in order to better understand the molecular dialogue between the protist and the plant. We analyzed the differential transcriptome between healthy and *P. betae* infected plants. The dynamic of the expression of some genes revealed by the differential transcriptomics was followed along the life cycle of *P. betae* in sugar beet.

The chapter 4 presents the study of the interactions between *P. betae* and plant defense ways using two complementary approaches performed in order to clarify the molecular pathways of plant defense expressed in

the presence of *P. betae*. The first was done by analyzing the response of *P. betae* infection after elicitation of the plant, and secondly by using *P. betae* as elicitor of defense ways against another sugar beet disease, Cercospora leaf spot.

A global discussion is presented in chapter 5. In this part, we present a model for the molecular interactions between *P. betae* and sugar beet. This model was based on results obtained from transcriptomics and plant defense ways analysis. According to this model, thesis results and a cost-benefit analysis, we discuss thereafter of the kind of parasitism exerted by *P. betae* on sugar beet. General conclusion and perspectives conclude the thesis, where we develop potential applications of the results of this thesis.

CHAPTER 1

LITERATURE REVIEW

1.1 Sugar beet cropping

Sugar beet cropping, from encouragement to limitation of production

The first *Beta* were probably grown at least 2000 years ago as a garden vegetable. This *Chenopodiaceae* was probably derived from various wild Mediterranean species such as *Beta maritima*, consumed widely throughout Europe from the Middle Ages as a spinach-like vegetable. The first field use of selected *Beta vulgaris* was as fodder for cattle, during the seventeenth century. From a white variety of fodder beet was developed the first sugar beet, with a relatively high sugar titer around 1800. The year 2011 was the "bicentenary of the sugar beet". The year 1811 indeed marked the beginning of the industrial culture of sugar beet. The spreading of this crop in Europe is particularly due to Napoleon, who encouraged sugar beet cropping in order to break the dependency of its empire on imports from Great Britain's colonies (Draycott, 2006). Two hundred years later, the old continent stays the most productive, with more than the half of the world production. France is the biggest world producer of sugar beet (31 millions of tons (MT)). The second biggest producer are the USA (31 MT) and the third, Russia (22 MT). Ten countries of the European Union (EU) are present in

the top 20 of sugar beet producers in which Belgium occupies the tenth place (http://faostat.fao.org). Since the establishment of the Common Agricultural Policy (CAP), quotas have been implemented in order to ensure prices higher than the world prices. This phenomenon can be observed in the fig. 1.1. In spite of the anger of European sugar beet growers in the view of the future suppression of theses quotas (foreseen in September 2015), the sugar beet cropping should probably persist in Europe due to the diversification of the uses of sugar (for bioethanol production e.g.). The production of sugar is now under the curve of world demand (fig.1.2).

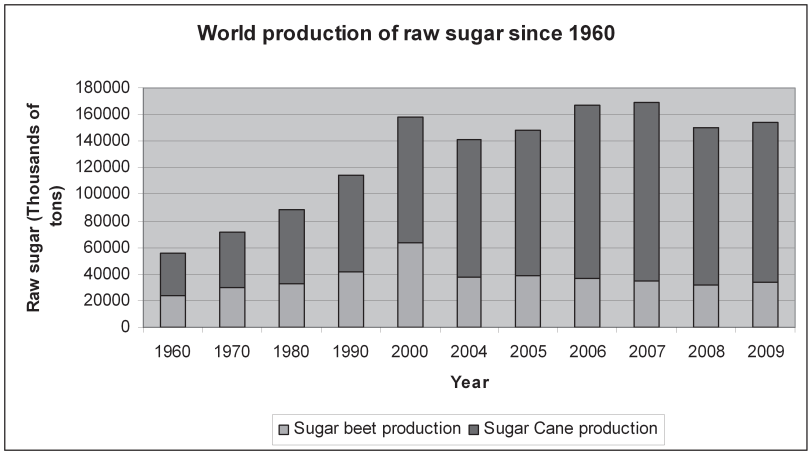


Fig. 1.1 : Part of sugar beet and sugar cane in world sugar production since 1960 (CGB (confédération générale des planteurs de betterave), 2011)

1.2 Sugar beet diseases

Sugar beet suffers from a lot of various diseases caused by pathogens from several kingdoms. In order to evaluate the most important diseases, the analysis of registered varieties and authorized pesticides in France and Belgium offers an original view of the economically important pathogens.

Registered varieties In the papers of ITB (2011) and Wauters (2012), varietal resistances against three diseases are present in the evaluated

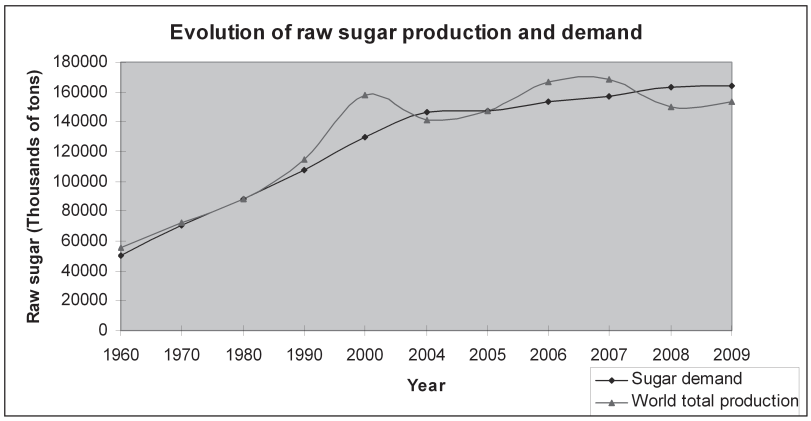


Fig. 1.2 : Evolution of production and demand of raw sugar (CGB (confédération générale des planteurs de betterave), 2011)

varieties: all analyzed varieties contain resistance against rhizomania, caused by the *Beet necrotic yellow vein virus* (BNYVV). Some varieties combine this resistance with a resistance to the cyst nematode (*Heterodera schachtii*) or Rhizoctonia root rot (*Rhizoctonia solani*).

Authorized pesticides According to an analyze of ITB[1] and fytoweb data[2], the available and registered fungicides on sugar beet are active against powdery mildew (*Erysiphe betae*), Cercospora leaf spot (*Cercospora beticola*), Ramularia leaf spot (*Ramularia beticola*) and sugar beet rust (*Uromyces betae*). In Belgium, Legrand & Wauters (2011) advise to use pesticides only if economic threshold is overcome. Other pathogenic fungi are not severe enough to be treated. Registered insecticides aim to kill aphids, vectors of severe viral diseases caused by *Beet yellows virus* and *Beet mild yellowing virus*.

The resistance to rhizomania is omnipresent in the current varieties grown in France and Belgium. This disease is indeed one of the most important diseases in sugar beet crops and is present in the majority of countries where sugar beet is grown. At the moment, rhizomania is

[1] www.itbfr.org
[2] www.fytoweb.be

present in Europe, North Africa, Asia and North America (McGrann *et al.*, 2009*a*), and probably keeps on spreading across the world (Rush *et al.*, 2006).

1.3 Rhizomania of the sugar beet

In 1952, poorly growing sugar beet crops were observed in northern Italy by Canova (1959). The damage to crops was so important that cropping of sugar beet was abandoned in this area. Due to its main symptom i.e. abnormal proliferation of dark and necrotic lateral roots, Canova (1966) named this disease "crazy root": rhizomania. The causal agent was discovered a few years later. Japanese scientists isolated, characterized and named the virus causing rhizomania: the *Beet necrotic yellow vein virus* (BNYVV) (Tamada *et al.*, 1971; Tamada & Baba, 1973). This name was given due to a secondary symptom, the necrotic yellow veins, sometimes observed on infected sugar beet leaves. However, most of the time, symptoms remain limited to the root system (Stevens *et al.*, 2006). Symptoms caused by rhizomania are illustrated in fig.1.3.
Since the 1960's, rhizomania has been spreading throughout Europe and the world. It is present in all sugar beet cropping areas in the world (Rush *et al.*, 2006).

The yield losses caused by rhizomania are due to two concordant events: the decrease of the size and sugar content of the taproot and the proliferation of lateral roots, which makes difficult the sugar extraction. The decrease of sugar yield can reach 80%, but is most commonly around 50% (McGrann *et al.*, 2009*a*). Two actors are principally involved in this pathosystem: the BNYVV and its vector, *Polymyxa betae*. Other viruses can be cotransmitted by *P. betae*: *Beet soilborne mosaic virus* (BSBMV), *Beet soilborne virus* (BSBV) and *Beet virus Q* (BVQ) (Crutzen *et al.*, 2009*a*,*b*; Wisler *et al.*, 1999).

1.3.1 BNYVV, the causal agent of rhizomania

Beet necrotic yellow vein virus is classified in the genus Benyvirus. It is the type species in this genus which contains another species, the *Beet soil borne mosaic virus* (BSBMV) (Tamada, 1999). BNYVV is composed by four (sometimes five) rod-shaped particles of 20 nm diameter

Fig. 1.3 : Rhizomania symptoms. a. Reduction of the taproot size of susceptible cultivars (right) in comparison with tolerant variety (left). b. Yellowing leaves of a row of susceptible sugar beets. c. Foliar symptoms: yellowing and necrosis of leaves veins. d. Proliferation of lateral rootlets (beard-like appearance of the taproot). e. Root symptoms: vascular system browning (vb) and root constriction (rc). f. Foliar wilt of infected sugar beets. (Courtesy of Yann Galein and Claude Bragard).

(Putz, 1977; Tamada *et al.*, 1989). These particles contain four or five positive RNA (ribonucleic acid) strands. The description of the BNYVV genome is showed in table 1.1.

Table 1.1: Genome organisation of BNYVV. The first RNA encodes for a polyprotein (replicase) containing methyl-transferase (MTR), helicase, protease and RNA-dependent-RNA polymerase domains. This RNA ensures the replication functions of the virus. The second RNA encodes for a coat-protein (CP) sometimes extended by a readthrough mechanism (CP-RT); protein of the Triple Gene Block (TGB) playing a role in the cell-to-cell movement, and the p14, involved in the suppression of the plant post-transcriptional gene silencing (PTGS). The RNA-3 encodes for three proteins, playing a role in pathogenicity and long distance movement (LDM). The p31 encoded by the RNA-4 plays a role in the transmission by *P. betae*, PTGS suppressor and pathogenicity. Finally, the facultative RNA-5 is principally involved in pathogenicity. Adapted from Hleibieh *et al.* (2007).

RNA	Proteins produced	Protein weight	Role
RNA-1	MTR domain Helicase Protease Polymerase	237 kDa (polyprotein)	Replication
RNA-2	CP	21kDa	Structure
	CP-RT	75 kDa	Structure, Transmission
	TGB-p1	42 kDa	Cell-to-cell movement
	TGB-p2	13 kDa	Cell-to-cell movement
	TGB-p3	15 kDa	Cell-to-cell movement
	p14	14 kDa	Suppressor of PTGS
RNA-3	p25	25 kDa	Pathogenicity
	N	6,8 kDa	Pathogenicity, LDM
	p4,6	4,6 kDa	unknown
RNA-4	p31	31kDa	Transmission Suppressor of PTGS Pathogenicity
RNA-5	p26	26kDa	Pathogenicity

Varietal resistance against BNYVV Up to now, the use of varietal resistance is the main strategy to control the BNYVV. Four resistance genes have been reported (Rz1 to Rz4), conferring tolerance or partial resistance to the virus. The virus titer in Rz1- or Rz2-containing sugar

beet cultivars is 6.10^4 times lower than in susceptible ones, Rz2 being considered as more resistant than Rz1. Rz3 offers a variable resistance and is probably allelic with Rz2. Rz4 confers partial resistance and is probably allelic with Rz1, Rz2 or Rz3 (Hleibieh *et al.*, 2007). The huge pressure imposed by the Rz resistance caused a rapid viral genetic drift, increasing the number of resistance breaking isolates (Acosta-Leal *et al.*, 2007). Another strategy is the use of transgenic sugar beets expressing viral RNAs encoding for coat-protein, replicase or movement protein. The impossibility for the virus to uncoat when CP is expressed or the effect of the PTGS (Scholten & Lange, 2000) are two alternative ways to decrease the titer in virus (Hleibieh *et al.*, 2007).

Phytoviruses transmission The viruses have to be disseminated to survive. About 10% of the viruses are able to be hosted in seeds of infected plants, allowing their through plant generations (Mink, 1993). Other very stable viruses can infect healthy plants with a simple contact between infected and healthy plants, as in the genus *Tobamovirus* and *Potexvirus*. Other viruses, such as *Dianthovirus* or *Tombusvirus*, persist in the soil and infect new rootlets by little wounds caused by root growth in the soil (Astier *et al.*, 2001). However, a large number of viruses are spread and transmitted by vectors. The BNYVV causes a soilborne viral disease. This virus is transmitted by the protist *Polymyxa betae* (Fujisawa & Sugimoto, 1977). Coinfection and multiplication in sugar beet roots of virus and vector ensures the meeting and thus the interaction protist-virus. This interaction provides to the virus a mean to be transmitted (by zoospores) and to survive (in resting spores of the protist). Three other viruses can be transmitted with or without BNYVV by *P. betae*: *Beet soil-borne mosaic virus* (BSBMV), implicated in the rhizomania complex in sugar beet, the *Beet soil-borne virus* (BSBV) as well as the *Beet virus Q* (BVQ) (Crutzen *et al.*, 2009*a*,*b*; Wisler *et al.*, 1999).

1.3.2 *Polymyxa betae*, the vector of BNYVV

***Polymyxa betae*, a plasmodiophorid** *Polymyxa betae* was first identified by Keskin (1964). This author classified the organism in the genus *Polymyxa*, which was described for the species *Polymyxa graminis* by

Ledingham (1939). Due to their similar morphology, these two species were for a long time distinguished only by the host range. The *Polymyxa* genus belongs to the Plasmodiophoromycetes family, first included in the Fungus Kingdom. This family contains ten genera and 35 species. Their common characteristics are the following: they are all obligate and intracellular parasites, their mobile stage is a biflagellated zoospore and they possess long-living resting spores. Moreover, their mitotic nuclear division is cruciform. The most known members of this family are phytopathogens and/or phytoviruses vectors. *Plasmodiophora brassicae* is the causal agent of the *Brassicaceae* clubroot and *Spongospora subterranea* causes powdery scab of potato and transmits the *Potato mop top virus*. *Polymyxa graminis* is the vector of numerous viruses mainly on cereals and other plants such as groundnut (Dieryck *et al.*, 2011), while *Polymyxa betae* transmits the BNYVV (Karling, 1968). After ultrastructural studies, Barr (1992) suggested that Plasmodiophorids should be considered as *Protozoa* rather than fungi. Recent studies have now precised the phylogeny of this group and Plasmodiophorids belong now in Phytomyxea, within Cercozoa, in the Rhizaria (Adl *et al.*, 2005; Bass *et al.*, 2009; Cavalier-Smith & Chao, 2003).

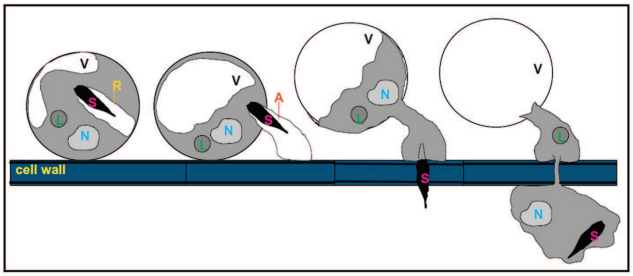


Fig. 1.4 : Diagram of *Polymyxa* zoospore encystment and penetration of the root cell. The inflation of the zoospore vacuole ejects a "needle" structure, the Stächel, which pierces the plant cell wall and enables the transfer of the zoospore content to the plant cell. V. vacuole. A. Adhesorium. L. Lipid droplet. N. Nucleus. R. Rohr. S. Stachel. V. vacuole. (Kanyuka *et al.*, 2003).

P. betae is an obligatory biotrophic parasite. Its life cycle is essentially realized in one host, mainly sugar beet (fig.1.5). It survives in the soil as sporosori (cluster of survival spores). Primary zoospores germi-

nate from sporosori and infect the root cells. After encystment of the zoospore on the surface of the host cell, the vacuole inside the zoospore inflates. The increasing pressure in the zoospore induces the ejection of a needle-like structure, the Stächel, which pierces the zoospore membrane and the plant cell wall. The subsequent inflation of the vacuole inserts the zoospore content inside the plant cell (fig.1.4)(Kanyuka *et al.*, 2003). The injected material from *P. betae* grows in the host cell in an amiboïd stage, the plasmodium. This plasmodium is a "vegetative" stage, in which a lot of nuclei are formed. These nuclei will be enclosed in zoospores or survival spores. During the sporangial phase, this plasmodium differentiates into a zoosporangium, an organ which ensures the mass production of secondary zoospores (Barr & Asher, 1996). The secondary zoospores infect new rootlets and, if they are viruliferous, can infect the plant with the BNYVV when releasing cytoplasm. During the sporogenic phase, the survival phase begins. The plasmodium differentiates into sporosori, clusters of resting spores. These spores can survive for years in the soil and, whether there are viruliferous, maintain the soil contaminated for a long time (Barr & Asher, 1996). The stages of the cycle are illustrated at the fig.1.6.

The BNYVV transmission

P. betae ensures the survival of the virus over years (by sporosori) and the transmission by zoospores (primary zoospores, from sporosori, or secondary zoospores, from zoosporangia). The mode of virus transmission by *P. betae* or by others plasmodiophorids was for a long time considered as passive: the virions are uptaken and released during the amembranous plasmodial phase of the plasmodiophorid (Campbell, 1996). But Tamada *et al.* (1996) showed that the readthrough protein of the coat protein of BNYVV played an important role in the transmission by *P. betae*, and its deletion could cause a significant decrease in the BNYVV transmission rates. Adams *et al.* (2001) showed that bioinformatic analysis of sequence data from viruses transmitted by plasmodiophorids (*Benyvirus*, *Bymovirus*, *Furovirus*, *Pecluvirus* and *Pomovirus*) strongly suggested that the transmembrane helices contained in the readthrough domain of the viruses CPs facilitate the movement of virus particles across the protist membrane. An active mechanism is then involved for

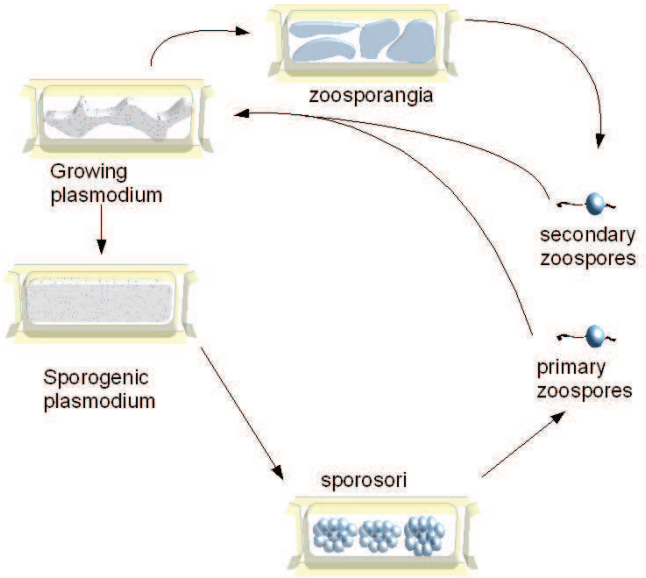


Fig. 1.5 : Life cycle of *Polymyxa betae*. A zoospore infects a sugar beet root cell by injecting its cellular content. A plasmodium grows in the root cell. Under favorable conditions, plasmodium differentiates into a zoosporangium, producing numerous secondary zoospores infecting new cell roots and producing plasmodia. When roots are heavily infected, or under defavorable conditions, plasmodia can differentiate into sporogenic plasmodia and further in sporosori. These spore clusters are able to remain alive for years in soil, in dormancy, waiting for a compatible host.

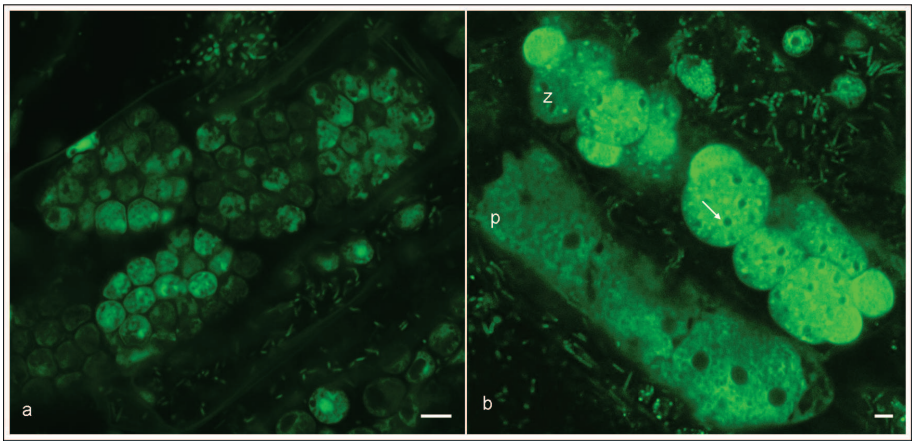


Fig. 1.6 : Stages of *Polymyxa betae*. a. Sporosori (survival spores) b. Zoosporangia (z) containing forming zoospores (arrow) and plasmodia (p). $Bars = 5\mu$.

acquisition and releasing of the virus by *P. betae*. Crutzen (2010) suggested that the *Polymyxa*-transmitted viruses entered the protist cell by budding, with a mechanism similar to the HIV: a "late-domain like" PSAP motif at C-terminal extremity of BNYVV CP-RT and putative ubiquitination site in the RT-domain supports this hypothesis.

Polymyxa betae as a second BNYVV host?

Until recently, the interactions between BNYVV and its vector were not characterized but considered as a "latent" stage of the virus, without multiplication nor replication in the vector. Recently, the eventuality that *P. betae* could act as an host of the virus too was brought up. Using immunofluorescence and immunogold labelling, Verchot-Lubicz *et al.* (2007) showed that BNYVV accumulates inside resting spores and zoosporangia of *P. betae*. Moreover, complementary strands of RNAs 1 and 2 of the BNYVV were detected by RT-PCR in *P. betae* zoospores inoculated with positive strands of RNA-1 and -2 by electroporation. These results suggest the replication of the virus inside the protist (Crutzen, F., Desoignies, N. & Bragard, C., unpublished). *P. betae* seems then to be an host of the BNYVV. But the mechanism of long-term survival of the virus inside resting spores and other stages of

the life cycle of the protist is not yet clear. In a similar pathosystem, the *Soil-borne wheat mosaic virus*, transmitted by *Polymyxa graminis*, Driskel *et al.* (2004) suggested that the virus could survive as ribonucleoproteic complexes.

1.4 Study of host-parasite molecular interactions

1.4.1 Plasmodiophorids-hosts molecular interactions: state of the art

Among the four Plasmodiophorids of agricultural importance, *Plasmodiophora brassicae* is the best studied (*P. brassicae* (319 papers), *Polymyxa betae* (129), *Spongospora subterranea* (112) and *P. graminis* (96))[3]. *Plasmodiophora brassicae* is not a vector of virus but is the causal agent of clubroot disease of *Brassicaceae*. Moreover, the fact that it is hosted by *Arabidopsis thaliana* and *Brassica napus*, two completely sequenced hosts, facilitated the plant-parasite interaction studies. Nowadays, recent papers provided investigations on cellular events leading to the gall formation (Malinowski *et al.*, 2012), on the role of ethylene signaling pathway in the reduction of root gall (Knaust & Ludwig-Müller, 2012) or on global gene expression of the host during the infection (Agarwal *et al.*, 2011). By an *in vitro* dual culture, genomics of *P. brassicae* are better understood (Bulman *et al.*, 2011). However, despite the availability of the complete genome sequences of two hosts, SSH is still used in other hosts to investigate interactions and discover new genes of *P. brassicae* (Sundelin *et al.*, 2011).

For the other plasmodiophorids, molecular plant-parasite interactions studies are scarce. For *Spongospora subterranea*, a dual culture on potato hairy roots was developed (Qu & Christ, 2007) and used in order to discover new genes of the organism and for plant hormone production studies (Bulman *et al.*, 2011). Some studies were done on *Polymyxa graminis*, principally with transcriptome expression analysis: the host and non-host response of the barley respectively against *P. graminis* and *P. betae* were analyzed using microarray. This study revealed that the early basal response was similar in both cases (McGrann *et al.*, 2009*b*). Another study was conducted in a similar way in order to determine

[3]bibliographic research made with Scopus, www.scopus.com

genes expressed during non-host response of sugar beet. This experiment was based on the representational difference analysis technique (McGrann *et al.*, 2007). Finally, a subtractive cloning of DNA from *P. graminis* was also conducted, but in a genomic view (Subr *et al.*, 2002).

1.4.2 Study models

Two models are commonly used in host-parasite interactions. *A. thaliana* is a useful model plant which offers numerous molecular tool, principally due to its little size and known genome. The second model is the use of hairy roots, i.e. individually growing roots, which enable the *in vitro* culture of obligatory parasites.

Arabidopsis thaliana

"Model organisms represent only a small fraction of the biodiversity that exists on Earth, although the research that has resulted from their study forms the core of biological knowledge" (Blair Hedges, 2002). Mendel was the first scientist to use pea as a model organism to study heredity. He defined outlines for the choice of model organisms (Müller & Grossniklaus, 2010). Afterwards, research communities focused on various models to highlight general principles underlying disciplines such as genetics, pathology and development. Before the "molecular era", model organisms were chosen for their short generation time and small size. At the beginning of the genome-sequencing projects, organisms with genomes of small size, as *A. thaliana*, were promoted. Nowadays, with the new sequencing techniques, the genome size is less determinant and economics often guide the choice of the model organism to sequence, especially for agricutural and human health research (Blair Hedges, 2002).

The history of *A. thaliana* as model organism is relatively recent. In 1976, at the second international symposium on *Arabidopsis*, only 25 researchers were present (Somerville & Koornneef, 2002). In 2011, the number of users of The *Arabidopsis* Information Resource (TAIR) reached 22,000 (Lamesch *et al.*, 2011). In the same way, the number of papers concerning *A. thaliana* (fig. 1.7) increased from 13 in 1985 to 2269 in 2011. Since the 1970's, a lot of events occurred, enhancing databases and tools based on *A. thaliana* and consolidating its use as

model organism. All discoveries and advances on *A. thaliana* are presented in the box 1 as a timeline. Thanks to the research communities, common databanks and tools such as TAIR, *A. thaliana* is now the best studied and most important model species for higher plants (Spannagla *et al.*, 2011).

Arabidopsis thaliana gets characteristics that make it amenable for scientific research. It is a small flowering plant and it is easy to grow a large number of them in controlled conditions. Its life cycle is short for a flowering plant, about 8 weeks, enabling to get several generations in a short time. Moreover each plant can produce a huge progeny (thousands of seeds), even after self-fertilization. This characteristic allows the rapid production of a large number of transgenic plants or mutants. In a genomic view, the genome of *A. thaliana* is one of the smallest genomes known in flowering plants. The availability of a lot of polymorphisms (50,000 between the two most used accessions) and ecotypes led to the discovery of genes functions and isolation of mutant genes. Consequently, discoveries triggering other ones, researchers using *A. thaliana* get at their disposal a large collection of mutants, ecotypes, transgenic and DNA microarrays with all genes known to be expressed. In the same way, databanks for genomics, functional genomics, transcriptomics, ... enriched every day (Somerville & Koornneef, 2002).

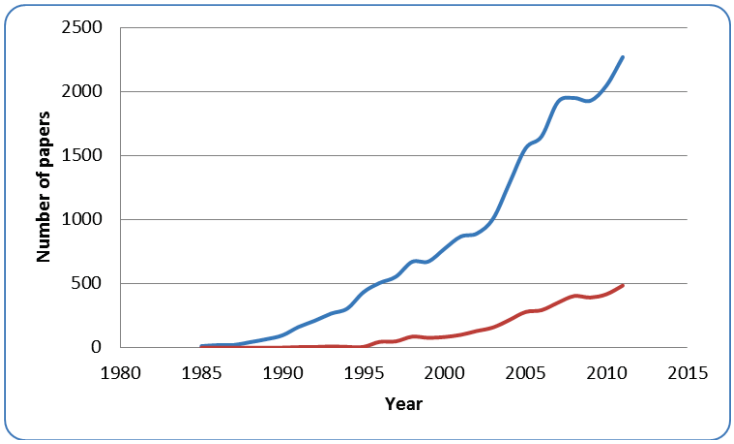


Fig. 1.7 : Evolution of the number of papers containing "*Arabidopsis thaliana*" (blue) or "*Arabidopsis thaliana*" and "Pathogen" (red) in title, summary or keywords. Research made using Scopus (www.scopus.com).

Box 1: *Arabidopsis* key events timeline

1907: *Arabidopsis thaliana* first use for experimental biology
1947: Report of first X-ray induced *Arabidopsis mutants*
1965: First *Arabidopsis* conference held
1983: First *Arabidopsis* genetic map
1986: First sequences published, First transgenic *Arabidopsis* plants
1988: Publication of the first *Arabidopsis* RFLP map
1990: Launching of *Arabidopsis* genome project
1993: High efficiency transformation developed
1994: Beginning of Expressed Sequence Tags (ESTs) sequencing
1997: Completion if physical maps of *Arabidopsis* chromosomes
1999: First DNA chips and microarrays available
2000: Completion of *Arabidopsis* genome sequencing
2002: DNA chip containing all known expressed genes of *Arabidopsis*
2003: First TIGR (The Institute for Genomic Research) *Arabidopsis* genome release
Source: adapted from Somerville & Koornneef (2002) and TAIR (www.arabidopsis.org).

In addition to providing precious tools to study evolution and plant physiology, *A. thaliana* is also often used, for the same reasons, as a model to study plant pathogen interactions. As shown on fig. 1.7, papers studying plant defenses and pathosystems using *A. thaliana* concern about 20% of the total of papers published these last years. The table 1.2 illustrates examples of studies in which *A. thaliana* is used as model for studying interactions with pathogens.

Table 1.2: Non-exhaustive list of pathosystems where *A. thaliana* is used as model organism

Organism	Interaction	Reference
Alternaria brassicola	Plant-Fungus	Pochon *et al.* (2012)
Phytoplasma	Plant-Phytoplasma	Maclean *et al.* (2011)
Plutella xylostella, Cotesia plutellae	Plant-Caterpillar-Parasitoid	Barker *et al.* (2007)
Botrytis cinerea	Plant-Fungus	Gonzalez *et al.* (2006)
Myzus persicae	Plant-Insect	Hunt *et al.* (2006)
Heterodera schachtii	Plant-Nematode	Wyss & Grundler (1992)
Plasmodiophora brassicae	Plant-Protist	Siemens *et al.* (2009)
Pseudomonas syringae	Plant-Bacteria	Innerebner *et al.* (2011)

Hairy roots

Two bacteria belonging to the family of *Rhizobiaceae*, *Agrobacterium tumefaciens* and *A. rhizogenes*, are exploited in research for their ability to transfect vegetal cells and to produce transgenic plants (Bevan & Chilton, 1982). These species are plant pathogens causing crown gall and hairy root disease, respectively. Plant inoculation with *A. rhizogenes* can give rise to hairy roots having phytohormone autonomy and showing accelerated elongation and branching (Kifle *et al.*, 1999). Their culture is easy and can be done under controlled conditions, in artificial media and *in vitro*. Hairy roots are very useful for several biotechnological applications (Veena & Taylor, 2007) :

- Their fast growth, short doubling time, easy maintenance and the possibility to produce a broad range of secondary metabolites make

the hairy roots more powerful than cell suspensions (Veena & Taylor, 2007; Hamill *et al.*, 1986).

- *A. rhizogenes* enables RNA interference (RNAi) allowing to study gene functions (Waterhouse & Helliwell, 2003). RNAi is also used to silence genes involved in interactions between root parasites and their hosts (pathogenic, commensal or symbiotic) (Collier *et al.*, 2005; Subramanian *et al.*, 2007).

- Hairy roots can facilitate the development of plants with tolerance to abiotic stress and are also useful in phytoremediation (Veena & Taylor, 2007). Moreover, hairy roots of hyperaccumulator plants are able to uptake heavy metals from polluted effluents, water or soil (Boominathan & Doran, 2003*a*,*b*).

- Hairy roots offer two ways to study plant-biotic interactions. The first is related to the study of gene expression: this tool has been used to study interactions between plants and nematodes, bacterias or mycorrhyzas (Mugnier, 1997; Narayanan *et al.*, 1999; Quandt *et al.*, 1993). The other way consists in the development of dual cultures, allowing to grow obligate parasites *in vitro*, under controlled conditions and in the absence of other microorganisms. These dual cultures have been developed for fungi (De Souza & Declerck, 2003), protists (Qu & Christ, 2007), bacteria (Veena & Taylor, 2007) and nematodes (Cho *et al.*, 2000).

1.4.3 Transcriptomic approaches

The differential transcripts analysis

The transcriptome can be seen as "a snapshot of the gene expression in a given cell or tissue at a given moment provided by capturing the total RNA within that tissue" (Ward *et al.*, 2012). The comparison of gene expression under different environmental conditions, treatments or pathogen attacks can help us to better understand physiological and molecular events in plants. Up to now, various techniques have been developed with different costs and sensitivities. They can be classified in three groups, the PCR-, sequencing- and hybridization-based technologies (Busch & Lohmann, 2007). The most known PCR-based tech-

nologies to study gene expression are the real-time quantitative reverse-transcription PCR (qRT-PCR) (Heid *et al.*, 1996) and the subtraction technologies (subtractive cDNA banks, representational difference analysis, ...) where the suppression subtractive hybridization is the most used technology nowadays (see below). A more recent sequencing-based technology, called SAGE, for Serial analysis of Gene Expression, consists in digestion of the total synthesized cDNA in short fragments (about fifteen nucleotides), concatenation of these short fragments and sequencing of concaneted tags (Velculescu *et al.*, 2000). RNA-seq is also a powerful tool, where a cDNA library constructed from the total mRNA population is sequenced using high troughput sequencing technologies (Wang *et al.*, 2009). Endly, the hybridization technology consists mainly in microarray analysis. This very powerful tool enables simultaneous detection of tens of thousands of transcripts at a reasonable cost (Busch & Lohmann, 2007). Sequencing- and hybridization-based technologies allow quantitative and exhaustive analysis of gene expression, but they require the whole genome knowledge of the analyzed organism. For non-completely sequenced organisms, SSH remains effective for a qualitative but less exhaustive analysis.

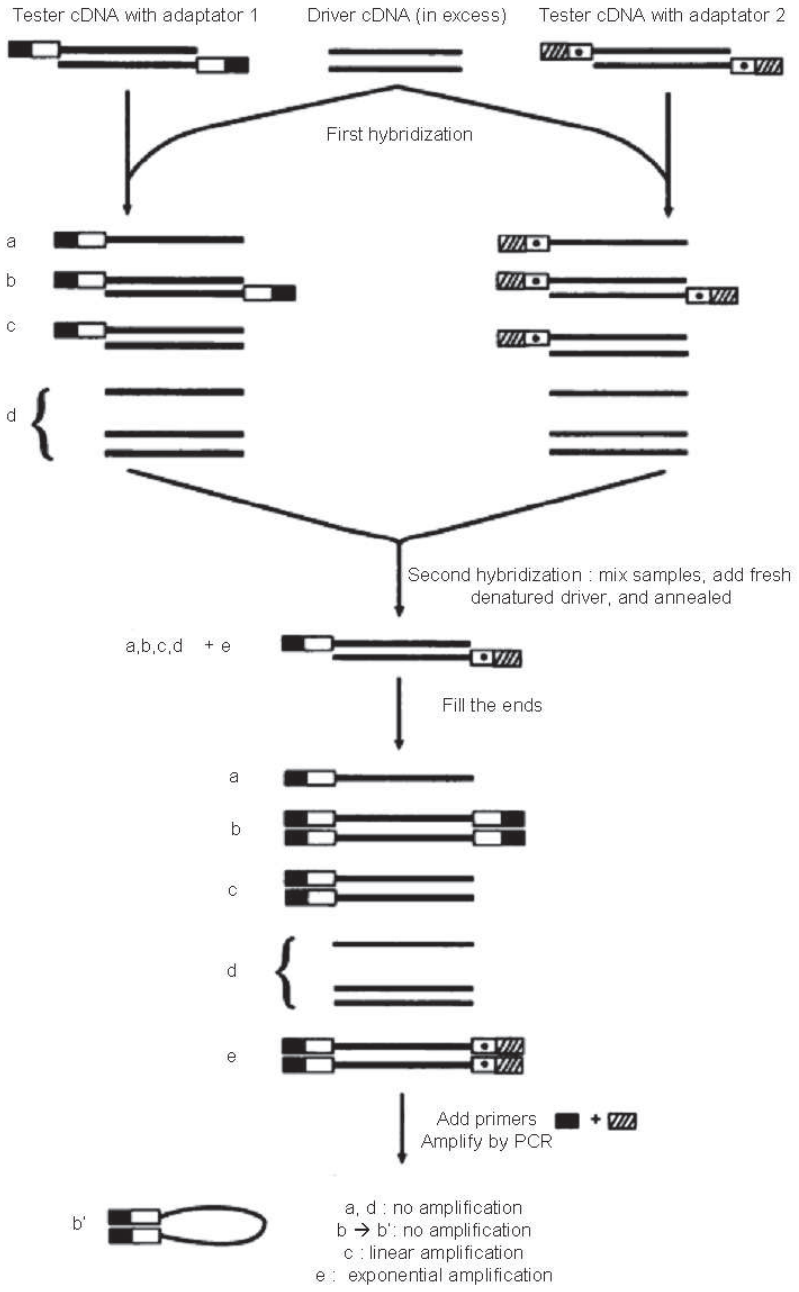
The suppression subtractive hybridization

The suppression subtractive hybridization was developed by Diatchenko *et al.* (1996). This technique can be seen as an evolution of the techniques of "subtractive cDNA hybridization" and "representational difference analysis". The principle of SSH is schematized at the fig 1.8. After extraction of the total RNA from two samples and transcription, the obtained cDNAs are digested with a restriction enzyme. The first difference with previous techniques is that the "tester cDNA" (constitutive cDNAs and cDNAs specific to the treatment) is divided in two pools and then ligated with different adaptators and thereafter denaturated. The denaturated "driver cDNA" (which will be subtracted from the tester cDNA) is added in excess to the two pools of testers, in order to hybridize with cDNA fragments common to the tester and the driver. After this first hybridization, a second hybridization is done by mixing the two first mixes and by adding denaturated driver in excess. The common fragments of the two tester pools absent in the driver can be

amplified by PCR using a pair of primers corresponding to the outer parts of the two adaptators (one primer per initial tester pool). The innovation in this technique is the "suppression" effect. This suppression is due to inverted terminal repeats which self-hybridize instead of hybridizing with the primer, and then decrease the amplification of false positives (Diatchenko *et al.*, 1996). After cloning of the resulting mix in a vector and sequencing, sequences corresponding to part of genes overexpressed in tester can be obtained. These little sequences, with a length between 200 to 900 base pairs (bp) and parts of coding sequences of expressed genes are called expressed sequence tags (ESTs).

SSH enables then sequencing of ESTs. Albeit ESTs represent only gene fragments, they are a very useful tool because these short sequences are a fast way to discover new genes (Bourdon *et al.*, 2002). Moreover, when the gene sequence is known, ESTs enable to discern genomic and coding sequences (Adams *et al.*, 1991). In addition to providing stuff for phylogeny studies, ESTs can also be useful in transcriptome analysis: if they can sometimes be interpreted directly for transcriptome activity, their principal advantage is to provide a basis for further gene expression analysis (Alba *et al.*, 2004).

Fig. 1.8 *(following page)*: Scheme of the suppression subtractive hybridization. After mRNA extraction, cDNA is synthesized, digested and adaptators are ligated (boxes). A first hybridization is done separately between the two different testers and an excess of the driver (negative control to be subtracted). The different possible combinations are shown. A second hybridization is then done by mixing together the solutions from the two first hybridizations and adding freshly denatured driver. After this double hybridization, a PCR is done with one primer corresponding to each adaptator. "a" fragments are only present in one pool of starting tester. They will not be amplified. "b" fragments are fragments hybridized from the same tester pool, the subtractive effect of the primers (self annealing of the fragment between the two adaptators). They will not be amplified. "c" fragments are fragments from one tester cDNAs hybridized with a fragment from driver cDNAs. Only a linear amplification will occur. "d" fragments, only present in the driver, will not be amplified. Only samples present in the two tester cDNAs and absent of the driver cDNAs ("e") will be exponentially amplified (Diatchenko *et al.*, 1996).


Tester cDNA with adaptator 1
Driver cDNA (in excess)
Tester cDNA with adaptator 2
First hybridization
a
b
c
d
Second hybridization : mix samples, add fresh denatured driver, and annealed
a,b,c,d + e
Fill the ends
a
b
c
d
e
Add primers
Amplify by PCR
b'
a, d : no amplification
b → b': no amplification
c : linear amplification
e : exponential amplification

1.5 The plant immune system

Animals counter pathogens with different barriers summarized in table 1.3. The first barrier is a physical one: skin and mucous membranes prevent the entry of microorganisms. Other non-specific defenses exist: phagocytosis and events such as inflammatory reaction or production of antimicrobial compounds can increase the general resistance rate of the animal. Finally, a specific, directed resistance occurs under action of lymphocytes and antibodies which specifically recognize pathogen cells and destruct them. This property is the one exploited with vaccines. Plants have an homologous immune system with non-specific and specific barriers (table 1.4). Cuticle and cell walls play the role of the physical barrier, with an ability to get thicker to increase the physical resistance. In parallel of the inflammatory responses, two systemic resistance ways are known in plants : the systemic acquired resistance (SAR) and the induced systemic resistance (ISR). These two resistance ways are induced by the interaction between the plant and a microorganism and act as an "aspecific vaccine". Their actions against pathogens are effective but less specific than mechanisms induced by gene-for-gene (GFG) interaction, producing proteins or molecules directed against the infecting pathogen. This last defense mechanism is very specific and enables a complete resistance of the plant to the pathogen.

Table 1.3: Outline of human defenses, based on Campbell & Reece (1995)

Non-specific mechanisms		Specific mechanisms
First defense barrier	Second defense barrier	Third defense barrier
Skin Mucous membrane	Phagocytes Antimicrobial compounds inflammatory reaction	Lymphocytes Antibodies

1.5.1 The zigzag model

The zigzag model is a model for the evolution of plant immune system and the sequential events in pathogenesis (Jones & Dangl, 2006). It is illustrated at the fig.1.9. Molecules common to many pathogens, i.e.

Table 1.4: Outline of plant defenses

Non-specific mechanisms		Specific mechanisms
First defense barrier	Second defense barrier	Third defense barrier
Cell wall Cuticle	Pathogenesis related-proteins Antimicrobial compounds ISR and SAR	Products of gene-for-gene relationship

the pathogen associated molecular patterns (PAMPs) are recognized by plants proteins, the pattern recognition receptors (PRRs). They elicit a moderate resistance response called PAMPs triggered immunity (PTI). In the second phase, a successful pathogen produces effectors interfering with PTI and which enable pathogen nutrition and dispersal: it is the effector triggered susceptibility (ETS). In the third phase, an effector is recognized by a plant receptor, often possessing a Leucine-Rich-Repeat (LRR) which induces an hypersensitive response (HR). It is the effector triggered immunity (ETI). The relation evolves then in a GFG interaction (Jones & Dangl, 2006). In addition to conferring a total resistance to the present pathogen, the HR induces one of the systemic resistance ways, the SAR, which will be active against future pathogen infections (see section 1.5.2).

The GFG defense system is based on the specific recognition of a pathogen protein by a plant protein. The pathogen protein, called Avirulence protein, is encoded by an Avr gene. The plant protein is called R (as resistance) protein. R proteins often possess a kinase activity. After activation, they induce an hypersensitive response, or hypersensitivity, and then an incompatibility of interaction.

1.5.2 The systemic resistance ways of the plants

The induced resistance can be defined as a "state of enhanced defensive capacity" (Van Loon *et al.*, 1998). The two most defined forms of induced resistance are the SAR and the ISR. These two forms can be differentiated on the basis of the elicitor nature and the regulatory pathways (Vallad & Goodman, 2004). The process of systemic resistance pathways can be divided in four steps (Pieterse *et al.*, 2009; Pieterse & Van Loon, 2004):

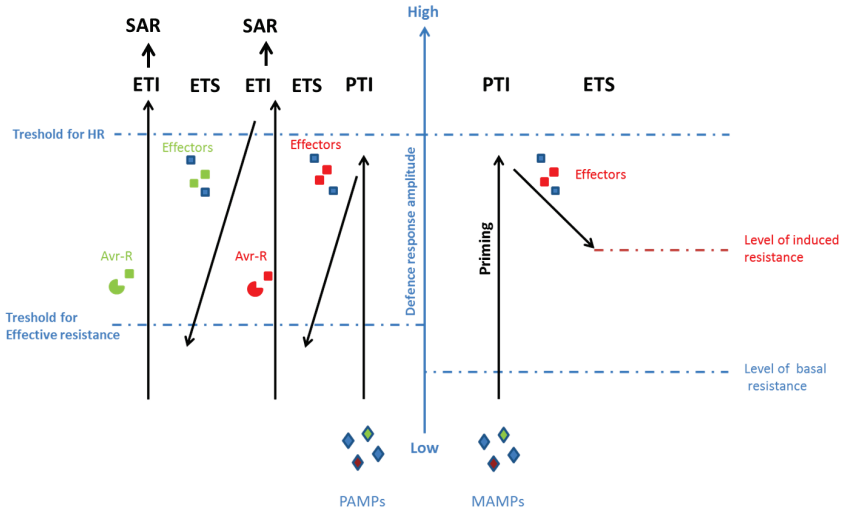


Fig. 1.9 : Zigzag model illustrating the quantitative output of the plant immune system adapted from Jones & Dangl (2006) and Van Loon (2009). On the left of y axis, the figure illustrates the zigzag model, where pathogen associated molecular patterns (PAMPs) confer a certain level of resistance (PAMPs triggered immunity, PTI), which can be reduced by pathogen effectors (Effector triggered susceptibility, ETS). These effectors can be recognized by the plant which can then induce an hypersensitive response (HR), resulting in effector triggered immunity (ETI). New effectors can be produced by the pathogen and new receptors can thereafter be synthesized by the plant. It is the zigzag model, also called "arm race". The systemic resistance ways are also illustrated in the figure. In the zigzag model, the systemic acquired resistance (SAR) can be induced by the HR. Induced systemic resistance (ISR) is illustrated on the right of the graph. After recognition of microbial associated patterns (MAMPs), a priming occurs. This phenomenon is not visible but will confer an enhanced resistance after subsequent pathogen attack.

1. the recognition of molecular pattern of a pathogen or plant growth promoting microorganism (principally bacteria or fungi),
2. the production of a signal molecule which travels throughout the plant,
3. the activation of a transcriptional factor, and
4. the defense response.

SAR

The SAR occurs after an infection by a pathogen. After recognition of Avr protein, an HR response can induce a resistance in the tissues surrounding the infection site. It is the local acquired resistance (LAR) (Conrath, 2009). This resistance can spread throughout the plant and become systemic. The SAR is associated with the production of PR proteins, which can have an antimicrobial activity and therefore contribute to the resistance (Van Loon *et al.*, 2006). At the cellular level, the accumulation of SA induces a redox potential modification activating the transcription factor NPR-1 (Jourdan *et al.*, 2008). If the role of SA accumulation in the inducing of SAR is accepted, the nature of the long distance signal molecule remains unclear. In the last years, some molecules have been suggested to be these signal molecules: methylsalicylate, methylated form of the salicylic acid, lipid-derived signaling molecules or reactive oxygen species (Conrath, 2009). During the SAR, the transcription activator NPR-1 induces the production of SAR-related compounds, the PR proteins.

The PR proteins were described as proteins only produced during pathogenesis. Others proteins often correlated with defense but sometimes detected in healthy tissues, such as phenylalanine ammonia lyase (PAL), were named pathogenesis related like (PRLs) proteins (Van Loon *et al.*, 1994). These proteins are now referred to as PR proteins, today defined as "all microbe-induced proteins and their homologues to the extent that enzymes which are generally present constitutively and only increased during most infections" (Van Loon *et al.*, 2006). The seventeen families of PR proteins are presented in the table 1.5. They exert a broad range of biological actions: chitinases which degrade hyphal walls,

Table 1.5: PR proteins families, adapted from Van Loon *et al.* (2006).

Family	Type member	Properties
PR-1	Tobacco PR-1a	Unknown, SAR marker
PR-2	Tobacco PR-2	$\beta - 1,3 - glucanase$
PR-3	Tobacco P,Q	Chitinases
PR-4	Tobacco 'R'	Chitinases
PR-5	Tobacco S	Thaumatin-like
PR-6	Tomato inhibitor I	Proteinase inhibitor
PR-7	Tomato P_{69}	Endoproteinase
PR-8	Cucumber chitinase	Chitinase
PR-9	Tobacco "lignin-forming peroxidase"	Peroxidase
PR-10	Parsley "PR1"	Ribonuclease-like
PR-11	Tobacco "Class V" chitinase	Chitinase
PR-12	Radish Rs-AFP3	Defensin
PR-13	Arabidopsis THI2.1	Thionin
PR-14	Barley LTP4	Lipid-transfer protein
PR-15	Barley OxOa	Oxalate oxidase
PR-16	Barley OxOLP	Oxalate oxidase-like
PR-17	Tobacco PRp27	Unknown

permatins, proteins facilitating the permeabilization of membranes or enzyme inhibitors... (table 1.5). A lot of PR proteins remain poorly known. Studies with transgenic plants expressing PR proteins are helpful to understand the mechanism of action of these proteins (Van Loon *et al.*, 2006).

ISR

The ISR occurs after contact between a plant and a non-pathogen microorganism, often a plant growth promoting rhizobacteria or plant growth promoting fungi. Phenotypically, ISR is similar to SAR: an infection of an elicited plant by a pathogen will produce less symptoms than on non-elicited plants. But the molecular mechanisms are different in both ways. The signalling molecules are different. In ISR, jasmonic acid (JA) and ethylene mediate the signal. The defense response is under the control of NPR-1 protein, as SAR, but interestingly, the response is different. In ISR, the defense response is called priming (Conrath, 2006).

The priming consists in an induced state, non perceptible in healthy

conditions, which allows the plant to develop more rapidly its defense mechanisms when a pathogen attacks. The system of priming allows a good protection of the plant with minimal costs: nothing is produced before the arrival of the pathogen (van Hulten *et al.*, 2006). Indeed, microarray analysis on *A. thaliana* did not show transcriptomic changes after the elicitation by a non-pathogenic *Pseudomonas fluorescens* strain and before an inoculation with pathogenic *Pseudomonas syringae*. On the contrary, after inoculation of the pathogen, transcripts corresponding to defense responses were directly visualized and overexpressed (Verhagen *et al.*, 2004). Priming is illustrated at the fig. 1.9.

1.5.3 Elicitors: to mimick natural events

Pathogens or non-pathogenic microorganisms possess patterns which can be recognized by the plants. The PAMPs and certain avirulence (Avr) proteins, as well as degradation products, can elicit a systemic response in the plant (Jourdan *et al.*, 2008). For non-pathogenic microorganisms, the known ISR elicitors are classified in three groups: cell surface components, metabolites under iron regulation and antibiotics (Ongena & Thonart, 2006).
With the discovery of the systemic resistance ways, the elicitors have been seen as new potential phytopharmaceutical products, acting like vaccines. Nowadays, a lot of PAMPs or microbial associated molecular patterns (MAMPs) are tested in order to evaluate their elicitation power. The table 4.1.2 presents recently discovered elicitors which are or could become future phytopharmaceuticals.

Table 1.6: Examples of different kinds and natures of isolated plant defense elicitors (adapted from Jourdan *et al.* (2008)).

Elicitor	Nature	Microorganism
Isolated from pathogens		
Coronatin	Bacterial toxin	*Pseudomonas*
Endopolygalacturonase	Fungal pectinase	*Botrytis*
Xylanase	Fungal enzyme	*Trichoderma*
Harpin	Bacterial membrane component	*Pseudomonas*, *Erwinia*
Avr protein		*Cladosporium*
Lipid transfer protein (LTP)	Oomycete LTP	*Phytophthora*
Oligogalacturonide	Parietal component of the plant	Product of pectic degradation
Synthetic analogs	BTH, SA	Analogs of signal molecules
Common molecules (Pathogens and PGPR)		
Lipopolysaccharides	Parietal components	*Xanthomonas*, *Pseudomonas*
Flagellin	Flagellar component	*Pseudomonas*, *Acidovorax*
Isolated from PGPR		
Pyoverdins	Siderophores	*Pseudomonas fluorescens*
Surfactin, Fengycin	Cyclic lipopeptides	*Bacillus subtilis*, *B. amyloliquefaciens*
N-Acyl Homoserine lactone	Quorum sensing molecule	*Serratia liquefaciens*

CHAPTER 2

EXPLORING NEW MODELS TO STUDY *POLYMYXA BETAE*

Opening comments

Two principal difficulties have to be overcome in view of molecular and genomic study of *Polymyxa betae*. The first issue arises from the fact that sugar beet is not a model organism: its genome is only partially known, making difficult the segregation between sugar beet and protist genomes. Secondly, the obligate endoparasitic nature of the protist makes the development of an axenic culture impossible. In order to overcome these difficulties, we developed two new study models presented in this chapter. The first paper concerns the finding of compatibility between *P. betae* and *Arabidopsis thaliana* by testing various ecotypes of the species. The second paper presents the development of a dual culture of *P. betae* enabling the production of zoospores in controlled media and conditions.

2.1 A new phenotype of *Polymyxa betae* in *Arabidopsis thaliana*

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Adapted from the paper published in *European Journal of Plant Pathology*[1].

2.1.1 Introduction

The understanding of the complex interactions between obligate parasites and their host plants benefits from development of model systems allowing to overcome the difficulties associated with their specific parasitism. *Arabidopsis thaliana* is one of the most studied model plants. The knowledge of the complete genome offers new molecular tools for studies of plant-pathogen interactions. The aim of this work was to develop an *Arabidopsis*-based experimental model that can offer new resources and tools to understand mechanisms involved in *Polymyxa* spp.-plant interactions. *P. betae* Keskin is a natural soil-borne parasite of the roots of *Chenopodiaceae* (Keskin, 1964). It is classified within the plasmodiophorids, a monophyletic group of obligate biotrophic parasites that includes 10 genera previously considered to belong to the Fungi, but which are now included in the Protozoa (Braselton, 2001). Its life cycle in the roots of *Beta vulgaris* is divided into four stages: after the infection of the host cell by a zoospore (the mobile stage of *P. betae*), a plasmodium grows in the cell and differentiates into a sporosorus (a cluster of resting spores) or into a zoosporangium, releasing secondary zoospores that are able to cause new infections, depending on the sporogenic or sporangial part of the cycle (Keskin, 1964). *P. betae* is economically important because of its capacity to transmit *Beet necrotic yellow vein virus*, the causal agent of rhizomania disease (Fujisawa & Sugimoto, 1977) and of three other viruses, *Beet soil-borne mosaic virus* (BSBMV) that is implicated in the rhizomania complex in sugar beet, the *Beet*

[1] *EJPP* 131:27-38 (2011)

soil-borne virus (BSBV) as well as the *Beet virus Q* (BVQ) (Crutzen *et al.*, 2009*a*,*b*; Wisler *et al.*, 1999). BNYVV and *P. betae* have a worldwide distribution (Peltier *et al.*, 2008) and the disease occurs in all major sugar beet growing areas. The incidence of rhizomania can be reduced by cultural practices, but the most effective control measure is the use of tolerant sugar beets, which limits virus replication and spread (Pferdmenges *et al.*, 2009; Rush *et al.*, 2006), but allows the viruliferous vector to remain in the soil. The development of cultivars resistant to *P. betae* would constitute an alternative to limiting vector multiplication and thus the production of viruliferous inoculum in the soil. Although resistance to *P. betae* development has been found in wild *Beta procumbens* and *B. patellaris* (Barr *et al.*, 1995), no cultivar of sugar beet with resistance derived from these wild species has yet been developed. An alternative approach to developing resistance to the vector would come from further knowledge (or studies) about the metabolic pathways involved in compatible and incompatible host-parasite interaction. However, the obligate nature of the parasitism and the relatively specific, restricted host range that allows the development of the sporangial and sporogenic phases of *P. betae*, complicate such studies. Knowledge about the genome of *P. betae* is still restricted. Parts of the ribosomal RNA, including the partial 18S ribosomal RNA gene, the internal transcribed spacer 1, the 5.8S ribosomal RNA gene, the internal transcribed spacer 2, and the partial 28S ribosomal RNA gene are sequenced (Bulman *et al.*, 2001; Legrève *et al.*, 2002; Ward & Adams, 1998). A few other sequences from *P. betae* are found in the NCBI database: a partial sequence of the mRNA for glutathione-S-transferase (Mutasa-Göttgens *et al.*, 2000), a RAPD-PCR amplified genomic *P. betae* DNA fragment, *P. betae* Keskin EcoRI genomic DNA fragment (Obermeier, 1998) and *P. betae* repetitive EcoRI-like fragments (Mutasa-Göttgens *et al.*, 1993). Knowledge of these sequences has permitted phylogenetic analyses of the species to be undertaken and development of specific molecular detection and quantification tools facilitating studies of this parasite. *P. betae* is considered as separate from *P. graminis*, a species with the same morphology parasitizing mainly monocotyledonous species (Barr, 1979) and differentiated into five formae speciales, depending on the specific combination of host range, temperature requirements and rRNA sequences (Legrève *et al.*, 2003, 2002; Ward & Adams, 1998). Apart from the *Polymyxa*

sequences, the known coding sequences of plasmodiophorids are limited to actin and ubiquitin genes (Archibald & Keeling, 2004; Bulman *et al.*, 2001; Ward & Adams, 1998), the trehalose-6-phosphate synthase gene from *Plasmodiophora brassicae* (Brodmann *et al.*, 2002) and 76 *P. brassicae* gene sequences obtained after suppression subtractive hybridization between RNA from *P. brassicae*-infected and uninfected *Arabidopsis* tissue (Bulman *et al.*, 2006). The host-*P. betae* molecular interactions and *P. betae* genes involved in the interaction are not well known. Only a few studies have been conducted on such interactions, i.e., comparisons between hosts and non-hosts (McGrann *et al.*, 2007, 2009*b*). Two species in *Brassicaceae* are known to be hosts of *P. betae* (Legrève *et al.*, 2005) whereas *Plasmodiophora brassicae*, another plasmodiophorid close to *Polymyxa betae*, infects a high number of species in *Brassicaceae*. As a first step for further molecular analyses and improving the knowledge of *P. betae* and host-parasite interaction genes, we propose to develop a new study model of *P. betae* by assessing the potential of infection and multiplication of a non-viruliferous isolate of *P. betae* in the model plant *Arabidopsis thaliana*, a member of *Brassicaceae*. During the past years, the tools available for *Arabidopsis* have been exploited in numerous studies on interactions between plants and pathogens. Microarray techniques permitted identification of key steps during the pathogenesis (Lee *et al.*, 2009; Siemens *et al.*, 2009; Yuan *et al.*, 2008), and mutants were used to study potentially interesting genes (Thatcher *et al.*, 2009). Studies of the development of *P. betae* in this model plant would allow the advantages of the model plant *A. thaliana* to be exploited. Distinct strategies and culture systems were tested to promote the four stages of *P. betae* within the roots of *A. thaliana*, with the focus of our approach lying in the confrontation between a *P. betae* monosporosoric isolate and a wide spectrum of *A. thaliana* ecotypes. A range of 14 ecotypes were tested for their capacity to establish compatible interactions with *P. betae*, by successive mass inoculations. The presence of *P. betae* in the roots of *A. thaliana* was subsequently assessed by a specific PCR assay and microscope observations.

2.1.2 Material and Methods

***P. betae* inoculum** The aviruliferous monosporosorus isolate A_{26-41} from a soil collected in a non-rhizomania-infested field at Opprebais in Belgium in 1987 was used in this study (Legrève *et al.*, 1998). The multiplication of this isolate was achieved by growing sugar beet (*Beta vulgaris* var. Cadyx) plants on a quartz-sporosori mixture using an automatic immersion system in environmental cabinets at 20-25°C, as described by Legrève *et al.* (1998). Large quantities of zoospores were produced in the roots of young plants growing in this system for 2-3 weeks, with flooding for 6 h every 12 h. After this period, tubes with *Polymyxa*-infected plants were removed from the system and placed for 24 h under non-saturated conditions in order to synchronize zoosporangial maturation. The roots of the sugar beet plants were then removed from the tubes, rinsed in sterile water and immersed in fivefold diluted Hoagland solution, at 4°C in order to stimulate the release of zoospores. The zoospore concentration of the suspensions was determined using a Thoma counting chamber.

Plants Fourteen ecotypes of *A. thaliana* of worldwide origin, able to grow under optimal conditions for *P. betae* (Table 2.1) and obtained from the European Arabidopsis Stock Centre, were tested for their compatibility with *P. betae*. Seeds were surface sterilized in 2.5% NaClO solution for five minutes and then rinsed three times in demineralised sterile water. Depending on the bioassay, sets of 4-5 seeds of each accession were sown in sterile quartz in six glass culture tubes (Figure 2.1) and in six tubes adapted to an automatic immersion system (Legrève *et al.*, 1998). The tubes were saturated with Hoagland solution and stratified for 48 h at 4°C. After this period, individual tubes were placed in a controlled environment room with a photoperiod of 12 h and at temperatures of 25-20°C. Four to five seedlings were obtained per tube. The sugar beet cultivar 'Cadyx' (rhizomania sensitive) was used as a positive control of *P. betae* infection and also for *P. betae* multiplication and source of inoculum. The plants were placed in the automatic immersion system and renewed every 2 weeks.

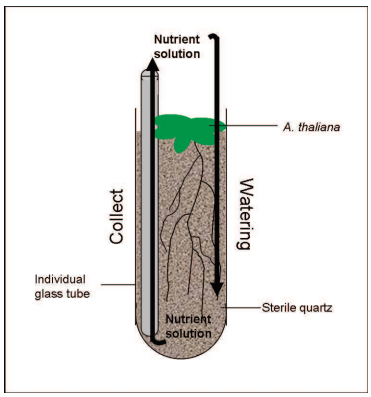

Fig. 2.1 : Individual glass tube system for *A. thaliana* culture. Seedlings are cultivated in sterile quartz and watered with Hoagland solution. The inner glass pipe allows to sample nutrient solution in order to detect *P. betae* zoospores.

Bioassays The *A. thaliana* seedlings were co-cultured in the automatic immersion system, with or without sugar beet infected with *P. betae*, or placed in individual tubes in sterile sand, depending on the intended bioassay. The automatic immersion system was placed in controlled environmental cabinets at 20-25°C, with a photoperiod of 12h. The automatic immersion system allowed alternate flooding-drainage periods (6 hours flooding/6 hours drainage) regulated by an electric timer. Three different bioassays were conducted to assess the establishment of *P. betae* infection in *A. thaliana*. First, the encystment of zoospores on *A. thaliana* roots of plants grown in individual glass culture tubes was examined by microscopy: five 3-week-old plants of the Col-0 ecotype were carefully removed from the tubes and immersed for 3 h in 1.5 ml of a 700,000 zoospores/ml suspension. The roots were then stained with the fluorescent lipophilic stain 3,3' dihexyloxacarbocyanine iodine $DiOC_6(3)$, following the method described by Barr *et al.* (1995). The $DiOC_6(3)$ was dissolved in 10% ethanol at a concentration of 0.5 mg/l and this stock solution was freshly diluted tenfold in milli-Q water before the roots were immersed in it for 15 min. The roots were rinsed in milli-Q water and observed by confocal microscopy, as described below. The susceptibility of the different ecotypes of *A. thaliana* to infection by *P. betae* was assessed in a second bioassay. Six tubes with 15-day-

old healthy plants of each *A. thaliana* accession and sugar beet (as the control) were placed in the automatic immersion system next to tubes containing sugar beet plants heavily infected with *P. betae* zoosporangia. Zoospores released from these plants into the nutrient solution served as inoculum to other plants in the system during the flooding periods. The root systems of two tubes per accession were harvested at 21, 33 and 45 days after inoculation (dai). The tubes containing *Arabidopsis* plants, which were removed from the system, were replaced by tubes with healthy sugar beet plants to promote the multiplication of *P. betae* in its host for a continuous production of zoospores. Tubes containing plants of each *A. thaliana* ecotype (one tube/ecotype) were also placed in the automatic immersion system in the absence of sugar beet and *P. betae* inoculum, as the negative control. After incubation, each root system was separated into two parts. *P. betae* infection was assessed by light microscopy for one part and by PCR for the second part. The infection of *A. thaliana* was scored by combining incidence and severity in the different ecotypes. The infection severity was assessed as described by Legrève *et al.* (2000): scores were given depending on the number of *P. betae* structures observed in roots, i.e., 0 if no structure is observed, 1 for 0-10 structures in the observed root system; 2 for 10 to 100 structures and 3 for more than 100 structures. The degree of infection was calculated for each accession over the times, as $\frac{\sum_{t=1}^{3}(Ninf_t \cdot S_t)}{Ntot}$, where $Ninf_t$ is the number of infected root systems at time t, S_t the infection severity at time t and Ntot the total number of tested root systems. A third assay was performed in order to assess the ability of each ecotype to release *P. betae* zoospores. *Arabidopsis thaliana* plants were sown in individual glass tubes filled with sterile quartz and containing a little glass pipe to allow drainage of the solution from the rhizosphere to the outer tube. The plants in each tube were inoculated three times, i.e., after 26, 33 and 45 days, with 40,000 zoospores in 5 ml at each inoculation time. Five tubes without plants were inoculated with the same suspensions and placed under the same conditions, as a negative control. In addition, plants from each ecotype were grown under the same conditions without inoculum, as a second negative control. The plants were watered with nutrient solution every 2 days. Six and nine days after the last inoculation, about 1 ml of the solution in each tube

was collected via the glass pipe and the presence of *P. betae* zoospores was examined by microscopy without staining, in order to observe the typical behaviour of the biflagellate *P. betae* zoospore and also after immobilization with iodine. The presence of zoospores was examined in the two samples. In order to ensure that the observed zoospores were those of *P. betae*, a specific PCR was performed directly on the solution, as described below. Furthermore, PCR was also performed on the roots, under the same conditions. The identification of organisms present in solution was done by amplifying and sequencing a part of the ribosomal DNA region including internal transcribed spacers (ITS) 1 and 2 using universal primers (ITS1 and ITS4).

Detection of *P. betae* in roots For light microscopy, roots were observed using differential interference contrast microscopy after lactophenol blue staining. For confocal microscopy, roots were stained with the fluorescent lipophilic stain 3,3' dihexyloxacarbocyanine iodine $DiOC_6(3)$ and analyzed using a Zeiss LSM 5 exciter (Carl Zeiss AG, Jena, Germany) confocal microscope, with a 488 nm excitation laser and a 505 nm barrier filter. For the immunolocalization of *P. betae* in roots of *A. thaliana*, roots were prepared and embedded in Technovit 8100 (Kulzer, Wehrheim, GE), following the manufacturer's protocol. Sections of 5 μm were cut and placed on a microscope polylysine slide in a water drop and warmed in a water bath. The slides were then incubated with 0.01% trypsin in $CaCl_2$, pH 7.8, for 5 min at 37°C. The specific immunolocalisation of *P. betae* was carried out as described by Ruzin (1999): the slides were incubated in a blocking solution (phosphate buffered saline with bovine serum albumin 3%, pH 7.2) at room temperature for 30 min, then rinsed with the PBS washing solution and treated with 10 μl of 100-fold diluted primary polyclonal antibody specific to *P. betae* and *P. graminis*, developed by Delfosse *et al.* (2000). After 90 min of incubation at 37°C, the slides were rinsed in PBS three times. Then 10 μl of 100-fold diluted Goat-Antirabbit (H+L) Cy3TM (LOEWE, Sauerlach, GE) was added to the slides, which were incubated at 37°C for 90 min. The slides were then rinsed twice in PBS for 15 min and in distilled water for 10 min. The slides were analyzed by confocal microscopy using the Zeiss LSM 5 Exciter with a 543 nm lazer, with a filter LP 560 nm and a beam splitter HFT 543 nm.

PCR detection Roots were removed from the automatic immersion system or individual tubes and were rinsed in demineralized water in order to eliminate possible external presence of *P. betae*. The total DNA of the root system was extracted using the FAST DNA© kit (Q.BIOGENE, Ca, USA) following the manufacturer's protocol. The roots were crushed in the CLS-Y buffer using the Fast PrepTM FP120 instrument (Q.BIOGENE, Ca, USA) for 40 s at speed 6. Extracted DNA was diluted 10 times in DEPC treated water. A volume of 2.5 μl of the tenfold diluted extract was tested in a total volume of 25 μl: each 25 μl mixture was prepared with 15.5 μl DEPC water, 2.5 μl 10X DNA polymerase buffer (Promega, Madison, USA), 2.5 μl of MgCl2 25 mM (Promega), 0.5 μl of each primer, 0.75 μl DNTPs and 0.25 μl of Taq DNA Polymerase (Promega) 5u/μl. The specific primers for *Polymyxa* detection, Psp1 (5'-TAGACGCAGGTCATCAACCT-3') and Psp2rev (5'-AGGGCTCTCGAAAGCGCAA-3'), developed by Legrève *et al.* (2003), were used to assess the presence of *P. betae* in the *A. thaliana* roots. The PCR was performed in an MJ Mini thermocycler (Bio-rad, Ca, USA). An initial denaturation was carried out at 94°C for 2 min, followed by 35 cycles, including 30 sec denaturation at 94°C, annealing at 60°C for 30 sec and elongation for 30 sec. A final elongation was completed at 72°C for 7 min. The samples were loaded onto an 1.2 % agarose gel in a Tris borate ethylenediaminetetraacetic acid buffer at pH 8 and the electrophoresis was performed using the Sub-Cell GT-Agarose Gel electrophoresis Systems (Bio-Rad). After migration, the bands were visualized using a Gel Doc 2000 (Bio-Rad).

2.1.3 Results

The potential infection of *A. thaliana* roots by *P. betae* was initially evaluated by testing the capacity of the zoospores released from sugar beet to encyst on seedling roots of ecotype Col-0. Three hours after contact between seedling roots and zoospores suspension, more than twenty zoospores were found encysted per *A. thaliana* root, mainly on the root hairs, as illustrated in figure 2.2.

The compatibility between *P. betae* and 14 accessions of *A. thaliana* was studied in a second bioassay in comparison with *B. vulgaris*, the most common host of *P. betae*. No *P. betae* was detected by PCR and

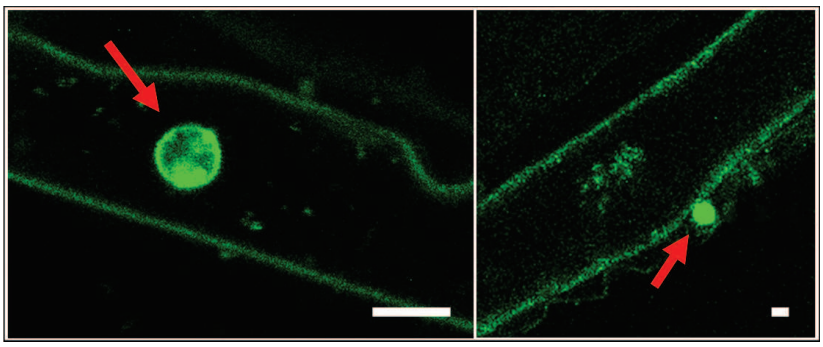

Fig. 2.2 : Zoospores encysted on an *A. thaliana* roots cells, observed after staining with $DiOC_6(3)$ with a confocal microscope. Bars = 5μ

no *Polymyxa*-like structures were observed in *A. thaliana* and *B. vulgaris* plants grown under the same conditions, in the absence of *P. betae* (data not shown). Twenty-one days after initiation of the co-cultures of *P. betae* and *A. thaliana*, *P. betae* infection was detected by PCR in eight accessions of *A. thaliana* (Table 2.1). Except for the Van-0 ecotype, this PCR detection was confirmed by microscopy. *P. betae*-like plasmodia and often resting spores, were observed in the *A. thaliana* roots (Table 2.1). The severity of infection was very low, with less than 10 infected cells for the Cvi-0 (N902), Bla-3, Bs-1, Kas-1 and Mh-0 ecotypes and between 10 and 100 infected cells for the Cvi-0 N1096 and Gr-1, and much lower than the severity of infection observed in *B. vulgaris*. One month after the co-culture was started, the infection was confirmed in these accessions, except for Mh-0, by PCR and microscopy. *P. betae* was present in one of two analyzed root systems for Cvi-0 (N902), Bla-3, Bs-1, Gr-1 and Van-0, with plasmodia and spore-like structures being observed. At this time, the presence of *P. betae* was also detected in Col-0 by PCR only. Two weeks later, *P. betae* was detected using both methods in all tested ecotypes except for Ita-0, the Moroccan ecotype. The severity of infection was higher than 15 days earlier for Cvi-0 (N902) and Bs-1, and remained high for Cvi-0 (N1096) and Gr-1. Plasmodia and resting spores were observed in almost all these accessions, except for Bd-0 and Kas-1, where only a few plasmodia were visible, and Bs-1, Kil-0 and Van-0, where only spores were detected. The degree of infec-

tion was calculated for each accession over the times in Table 2.1 (Figure 2.3). Although *P. betae* infection was detected in all but one accession, differences in the degrees of infection, depending on the accessions, were observed, with the highest values for ecotype Cvi-0 (N1096) and the lowest for Kil-0 (UK), Mh-0 (Poland) and Van-0 (Canada). The infection degree depends on the incidence and severity of infection, and provides information on the compatibility of the host-parasite interaction. For example, *P. betae* was detected in root systems of accessions Co-2 (Portugal) and Cvi-0 (N1096) (Cape Verde), but the degree of infection in Cvi-0 was two times higher than in Co-2.

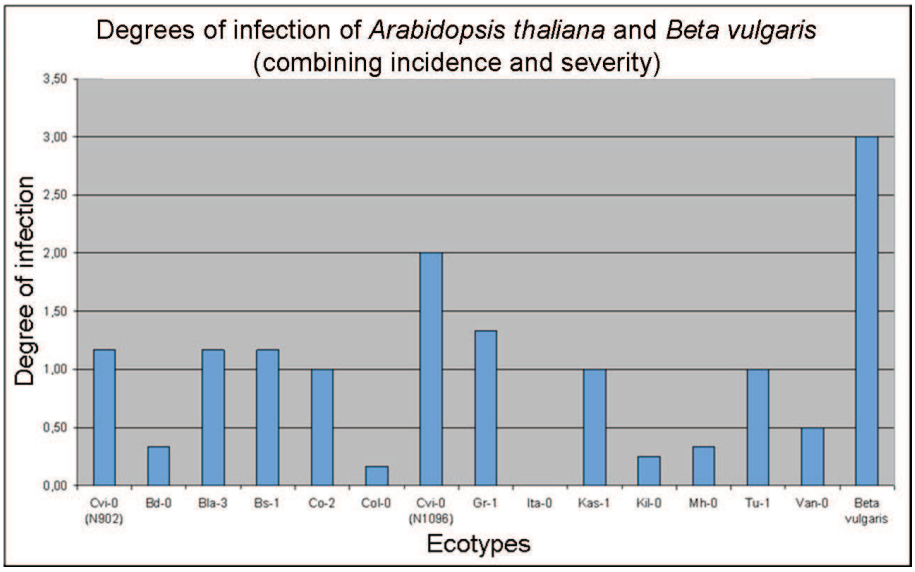

Fig. 2.3 : Degree of infection of 14 ecotypes of *A. thaliana* and of *B. vulgaris* by *P. betae*. Ecotypes compatibility (degrees of infection) was calculated by combining the susceptibility (the ratio of the infected plants on tested plants) and degree of infection, depending on the quality of infection (number of cells infected).

Table 2.1: Detection of *P. betae* infection in *A. thaliana* ecotypes and *B. vulgaris* after three periods of incubation[A]

Ecotype	Origin	21 dai			33 dai			45 dai		
		PCR[B]	Microscopy[C]		PCR[B]	Microscopy[C]		PCR[B]	Microscopy[C]	
			S	OBS		S	OBS		S	OBS
A. thaliana										
Cvi-0 (N902)	Cape Verde Islands	1/2	+	P	1/2	++	P,S	2/2	++	P,S
Bd-0	Germany	0/2	-	-	0/2	-	-	2/2	+	P
Bla-3	Spain	2/2	+	P,S	1/2	+	P,S	2/2	++	P,S
Bs-1	Switzerland	2/2	+	P,S	1/2	+	P,S	1/2	++	S
Co-2	Portugal	0/0	nt	nt	0/0	nt	nt	2/2	++	P,S
Col-0	USA	0/2	-	-	1/2	-	-	1/2	+	P,S
Cvi-0 (N1096)	Cape Verde Islands	2/2	++	P	2/2	++	P,S	2/2	++	P,S
Gr-1	Austria	2/2	++	P,S	1/2	++	P,S	1/2	++	P,S
Ita-0	Morocco	0/0	nt	nt	0/2	nt	nt	0/2	-	-
Kas-1	India	2/2	+	P,S	2/2	+	P,S	2/2	+	P
Kil-0	UK	0/0	nt	nt	0/2	-	-	1/2	+	S
Mh-0	Poland	1/2	+	P,S	0/2	-	-	1/2	+	P,S
Tu-1	Italia	0/0	nt	nt	0/0	nt	nt	2/2	+	P,S
Van-0	Canada	1/2	-	-	1/2	+	P,S	2/2	+	S
B.vulgaris	SES Vanderhave	2/2	+++	P,Z,S	2/2	+++	P,Z,S	2/2	+++	P,Z,S

[A] for Col-0 the different times were 15,30 and 45 days after inoculation (dai) [B] Detection by PCR : number of infected roots systems (Ninf) out of number of tested roots systems (Nt) [C] Detection by light microscopy: severity of infection (S) was evaluated by counting the total number of *P. betae* structures observed in root systems : (-) no structures; (+) 0-10 structures; (++) 10-100; (+++) more than 100 structures; nt - not tested. The observed stages of the life cycle are annotated as following: P - plasmodia ; S - Sporosori ; Z - zoosporangia.

In *B. vulgaris*, the degree of infection increased over time and exceeded 1,000 infected cells 21 days after initiation of the co-culture between *P. betae* and *B. vulgaris*. In the roots of *B. vulgaris*, the three life stages of *P. betae* (formation of plasmodia, zoosporangia and sporosori) were visible in all the observed root systems, with the sporangial part of the cycle being predominant at the first observation date and the sporogenic part of the cycle at the later dates. As shown in Table 2.1, no zoosporangia was observed in the root system of *A. thaliana*, whatever the accession. Although the shapes of the observed plasmodium structures in *A. thaliana* and *B. vulgaris* root epidermis cells or root hairs look very similar (Figure 2.4), the resting spore arrangement was clearly different, depending on the host plant species. In *B. vulgaris*, the spores were grouped in clusters of tens to hundreds of spores. In *A. thaliana*, the resting spores were similar in size and morphology to the spores produced in *B. vulgaris*, but the spores appeared in the root cortical cells either isolated or grouped along a string or in a loose cluster (Figure 2.4). The confirmation of the identity of these spores observed in light microscopy as *P. betae* spores was obtained by immunodetection using a *Polymyxa*-specific antibody revealed by confocal microscopy (Figure 2.5).

In *A. thaliana*, no typical zoosporangia were observed. In order to check the ability of *P. betae* to produce its sporangial phase in *A. thaliana*, the release of zoospores from *A. thaliana* roots was tested in a third bioassay by analyzing the presence of zoospores in the solution surrounding the roots of living *A. thaliana* inoculated with *P. betae* and grown in the glass culture system. Bi-flagellate zoospores were detected at 6 and/or 9 dai in the solution surrounding living plants of all *A. thaliana* accessions, except for Bd-0 and Gr-1. The identification of these zoospores as *P. betae* zoospores was confirmed by specific PCR and ITS sequencing. To confirm that the zoospores observed in the solution surrounding the plant roots were not the originally inoculated zoospores, a control test was performed on the solution collected in three tubes inoculated with the same number of zoospores, but without plants. No zoospores could be detected in these control tubes.

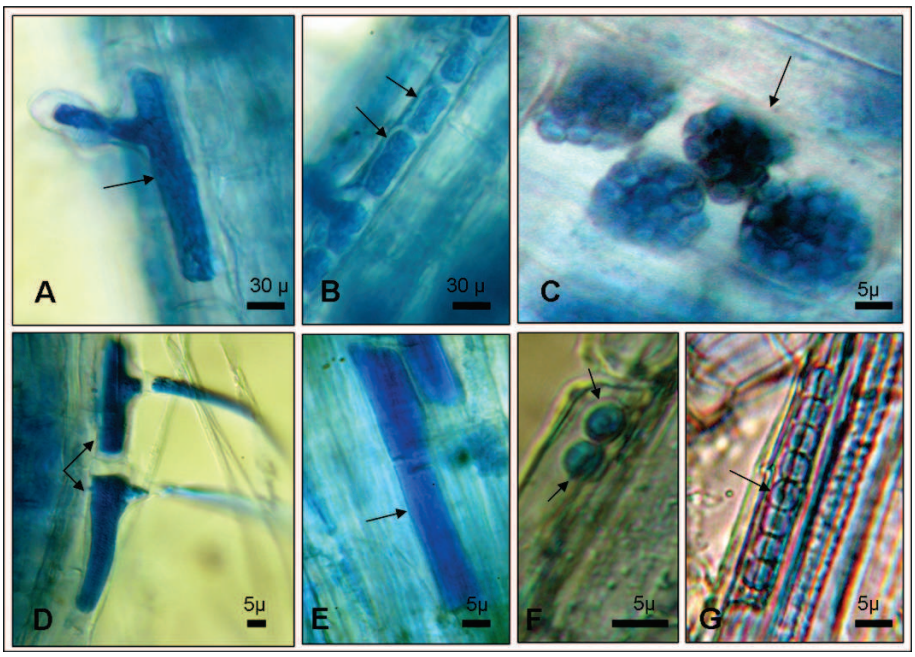

Fig. 2.4 : Different structures of the life cycle of *P. betae* on sugar beet (A-C) and *A. thaliana* (D-G) roots. A,D) Plasmodia. B) Zoosporangia. E) Differentiating plasmodium. C,F,G) Resting spores, gathered in clusters (C) or in chains (F,G).

2.1.4 Discussion

Compatible interactions between *P. betae* and *A. thaliana* would be a useful tool for understanding the molecular interaction between *P. betae* and its host by providing a system for identifying both host and parasite genes induced during infection. Although the host range of *P. betae* was first considered to be restricted to *Chenopodiaceae* (Keskin, 1964), studies have shown that species from two other families, *Amaranthaceae* and *Caryophyllaceae*, might also be heavily infected (Abe & Ui, 1986; Barr, 1979; Barr & Asher, 1992). More recently, molecular tools have allowed *P. betae* to also be detected in some *Asteraceae*, *Papaveraceae*, *Poaceae* and *Urticaceae*, as well as in two species in *Brassicaceae*, i.e., *Capsella bursa-pastoris* and *Thlapsi arvense* (Legrève *et al.*, 2005). Although *P. betae* has not yet been reported to infect *A. thaliana*, the

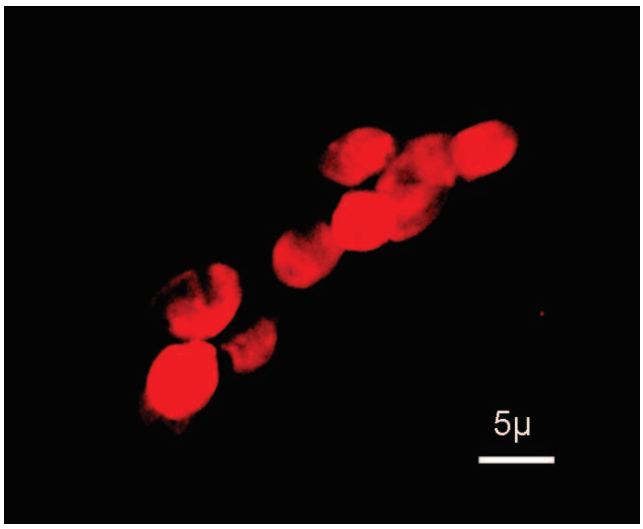

Fig. 2.5 : Confirmation of the identification of *P. betae* resting spores in *A. thaliana* roots by immunolocalisation using a *Polymyxa*-specific antibody.

detection of this parasite in roots of *Brassicaceae* and the high rate of multiplication of other plasmodiophorids (*Plasmodiophora brassicae*) on *A. thaliana* (Desoignies *et al.*, 2009; Koch *et al.*, 1991) led us to assess the compatibility between *P. betae* and distinct *A. thaliana* ecotypes under optimal conditions for the development of *P. betae*, using a monosporogenic isolate of *P. betae* as inoculum.

We observed that zoospores are able to detect the presence of *A. thaliana* roots and to encyst on them. This stage of the infection does not appear to prevent establishment and development of *P. betae* on *A. thaliana*. This result is in accordance with the observations of Barr *et al.* (1995), who observed encysted *P. betae* zoospores on *Beta patellaris* and *B. procumbens* roots despite lack of further infection in these species.

Polymyxa betae was detected in all but one of the 14 tested ecotypes, using PCR as well as microscopy. The differences in the degree of infection and in the observed stages, depending on the *A. thaliana* accessions, revealed differences in compatibility. The higher degree of infection observed for ecotypes Cvi-0 (N1096) and Gr-1 suggests a higher degree

of compatibility than for the other ecotypes. Especially the ecotypes Bd-0, Col-0, Kil-0 and Mh-0 harboured a lower degree of infection. The absence of sporosori in the roots of the ecotypes from the Cape Verde Islands [Cvi-0 (N902) and Cvi-0 (N1096)] at 21 dai, on which only plasmodia were present, combined with a relatively high degree of infection at 33 and 45 dai, could suggest that the sporangial phase of *P. betae* is promoted in these ecotypes rather than the spore-forming phase, at least during the first 3 weeks after inoculation. In contrast, the detection of sporosori in the less susceptible Bs-1, Van-0 and Kil-0 ecotypes 45 dai revealed that the sporogenic phase was already initiated in them. The factors determining the sporangial vs sporogenic development from the plasmodial stage still remain unknown (Braselton, 1995; Littlefield *et al.*, 1998). However, from the dynamics of the development of sporangia and sporosori observed for *P. betae* on sugar beet and for *P. graminis* on cereals (Legrève *et al.*, 1998), the sporangial phase appeared to occur in environments favourable to the multiplication of *Polymyxa* sp. In contrast, the survival stage was initiated when the infection became high or the conditions were not optimal. This behaviour has been reported for other soil microfungi (Grishkan *et al.*, 2003) and could explain why, in a less susceptible host, the sporogenic phase is activated earlier than in a compatible interaction. Interestingly, the ecotype most compatible with *P. betae* [Cvi-0 (N1096)] is also known to be particularly susceptible to the infection and the multiplication of *Plasmodiophora brassicae* (Kobelt *et al.*, 2000). At the morphological level, our observations indicated that the morphology of *P. betae* in *A. thaliana* is distinct from the *P. betae* structures observed in the roots of *B. vulgaris*. As shown in Figure 2.4, the plasmodia formed on both species are similar except that the plasmodial dimensions are smaller in *A. thaliana* than in sugar beet. The size of the plant cells in the two distinct plant species could influence the size of the plasmodia. In addition, the morphology of sporosori in these two species is different: in sugar beet, *P. betae* resting spores are grouped into clusters of numerous spores to form sporosori whereas in *A. thaliana*, only a few individual resting spores are present in a cell, sometimes forming strings or disconnected groups of a few resting spores. The observation of particular phenotypes of *P. betae* resting spores, being dependent on the host cell, is in accordance with reports by Barr (1979) who also observed distinct forms of *Polymyxa* spp. in different

host plants, and concluded that the resting spore clusters might assume the shape of the host cell. It is interesting to note that the morphology of *P. betae* spores, shown in Figure 2.4 on *A. thaliana*, is very close to the morphology of *P. brassicae* resting spores produced on the same plant (Koch *et al.*, 1991). This phenotype might be related to a specific interaction between two plasmodiophorid species and *A. thaliana*. Although no typical zoosporangia could be identified in the roots of *A. thaliana*, the observation of zoospores in the nutrient solution surrounding the rhizosphere for all accessions except ecotypes Bd-0 and Gr-1 showed that zoospores were produced and thus zoosporangia present. The low number of zoospores indicated that zoosporangia are scarce in *A. thaliana* compared with sugar beet, even in susceptible accessions of *A. thaliana* in which the degree of infection is considerably lower than in *B. vulgaris*. The absence of zoospores for ecotypes Bd-0 and Gr-1 reflects the absence of zoosporangia in their roots at the time of the analysis. Zoospores were detected in the rhizosphere of the Moroccan ecotype Ita-0 and *P. betae* was detected in plants grown in the glass culture tube system, although no infection was detected in plants grown and inoculated in an automatic immersion system. This discrepancy could be explained by the differences in culture systems between the automatic immersion system and the individual tubes, or by the limited number of tested plants of this ecotype due to the low germination rate. The lack of detection of typical zoosporangia in the roots of *A. thaliana*, although zoospores are produced, could be explained by differently shaped zoosporangia in the roots of this species. The small size of the cells might inhibit the division of zoosporangia into several segments, as usually observed on sugar beet, and makes it difficult to distinguish between the plasmodial and zoosporangial stages. The different levels of granularities observed for plasmodia in the microscope observations could be linked to the degree of differentiation of zoosporangial plasmodia: a higher level of granularity could correspond to zoosporangia. The plasmodial structures shown in Figure 2.4 could correspond to *P. betae* zoosporangia. The ability of *P. betae* to re-infect sugar beet after one life cycle was not tested, but Legrève *et al.* (2005) showed that inoculum from some alternative hosts could re-infect sugar beet. It would be interesting to test the ability of *P. graminis* to develop on *A. thaliana*. Indeed, both pathogen species are close genetically (Legrève *et al.*, 2002; Ward & Adams, 1998) and *P.*

graminis has been shown to infect some dicotyledonous species (Legrève *et al.*, 2000). We obtained compatible interactions between *P. betae* and *A. thaliana* under conditions favourable to *P. betae* growth and found differences in *P. betae* infection degree between the tested ecotypes. These results may be used in the development of a new model system to study interactions between *P. betae* and its hosts. The ecotype Cvi-0 appeared to be particularly interesting in the development of this new model system because *P. betae* infection in this ecotype was higher than in the others ecotypes. The *P. betae* genome is still largely unknown because of the obligate nature of this parasite and because the complete genome of sugar beets is not known. Use of the *A. thaliana*-*P. betae* interaction as a new model will allow the use of the molecular tools available for this plant to investigate mechanisms underlying plant-parasite interactions. A transcript analysis of differentially expressed genes after *P. betae* infection will be possible. Identification of *P. betae* genes will be possible using a bioinformatics approach. Separation of host and parasite genes will be possible since *A. thaliana* is full-genome sequenced. However, *P. betae* infection in roots of *A. thaliana* is lower than in the natural host and the phenotype is slightly different, indicating that this plant-parasite interaction may be host-specific. Therefore, even though this model offers new possibilities, it should be considered as complementary to the *P betae-B. vulgaris* system.

2.2 In Vitro Dual Culture of *Polymyxa betae* in *Agrobacterium rhizogenes* Transformed Sugar Beet Hairy Roots in Liquid Media

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2.2.1 Introduction

Polymyxa betae Keskin is a soil-borne parasite of roots of *Chenopodiaceae* (Keskin, 1964). It belongs to the *Plasmodiophorida*, a monophyletic group of obligate biotrophic parasites comprising 10 genera and now included in the *Cercozoa*, *Phytomyxea* (Adl *et al.*, 2005). Unlike other plasmodiophorids, such as *Spongospora subterranea* and *Plasmodiophora brassicae*, causal agents of powdery scab on potato and clubroot in *Brassicaceae* respectively, *P. betae* is asymptomatic. However, it is an important vector of *Beet necrotic yellow vein virus*, the causal agent of rhizomania disease (Abe & Tamada, 1986; Fujisawa & Sugimoto, 1977). The obligate nature of this parasite prevents the possibility of an axenic culture of this organism. Recently, dual cultures on hairy roots have been developed for some plasmodiophorids (Asano *et al.*, 1999; Qu & Christ, 2007) and other biotrophic organisms, such as arbuscular mycorrhizal fungi (De Souza & Declerck, 2003). A dual culture system for *P. betae* in sugar beet hairy roots has been developed and is described in this paper.

2.2.2 Material and Methods

Source of sugar beet hairy roots Hairy roots were developed from 1-month-old sugar beet plants (cultivar Cadyx). The K599 strain of

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Agrobacterium rhizogenes was obtained from the National Collection of Plant Pathogenic Bacteria Central Science Laboratory, Sand Hutton, York YO41 1LZ England. A suspension of this strain was prepared from 48 h-old colonies grown on a nutrient agar medium at 25 °C (Qu & Christ, 2007). The colonies were suspended in 5 mL of sterile water containing 0.85% (w/v) NaCl. The petioles were disinfected for 20 min in a 2.5% (w/v) $Ca(ClO)_2$ aqueous solution, cut into 2 cm-long pieces. These petioles were soaked in the bacterial suspension, grown on Gamborg's B5 medium (Gamborg *et al.*, 1986), and kept in the dark at 25 °C. After one week, the emerging transformed roots were cut and placed on a Gamborg B5 medium with sucrose (30 g/L) and cefotaxime sodium (200 ppm). Three subcultures were carried out over a 2-week period in order to completely eliminate *A. rhizogenes*. The hairy roots were then maintained on the same medium without cefotaxime.

Inoculation of hairy roots The monosporosoric aviruliferous *P. betae* isolate A_{26-41} (Legrève *et al.*, 1998) was multiplied on sugar beet roots. Sporosori of the protist were inoculated on 3-week-old seedlings placed in an automatic immersion system with a 12-h photoperiod (20 and 25°C) and 6-h cycles of irrigation and desaturation (Legrève *et al.*, 1998). After three weeks, typical zoosporangia were observed in the roots by light microscopy. The roots were then cut into 3 cm-long pieces and disinfected for 10 min in a 4% (w/v) $Ca(ClO)_2$ solution. These root pieces were rinsed three times in sterile milliQ water, and 20 of them were each placed in a drop of sterile water (pH 7.2) in the presence of one sugar beet hairy root about 3 cm in length and incubated for 24 h in sterile conditions. The presumably infected hairy roots were transferred to a Gamborg B5 liquid medium supplemented by sucrose (1g/L) and at pH 7.2, the optimal pH for growth of the protist (Goffart & Maraite, 1991). Each hairy root was placed with 5 mL of the medium under sterile conditions in a 20-mL syringe capped by a 0.20-μm Acrodisc filter (Pall, Port Washington NY, USA), which allows gas exchange while preventing external contamination (fig. 2.7). The liquid nature of the medium and the low concentration of sucrose allowed zoospore mobility. The hairy roots in syringes were incubated without shaking in the dark for 10 weeks for 12-h periods at 18 °C and 23 °C respectively.

Hairy roots analysis After 10 weeks, a time sufficient for *P. betae* to complete several life cycles, the transformed hairy roots were analyzed using light and confocal microscopy and a specific PCR. Staining of the *P. betae* structures in the roots was done by boiling the root samples for 2 min in lactophenol blue or by incubation of other root samples in 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6(3)$), following the methods reported by Barr *et al.* (1995). Light microscopy was used to highlight typical plasmodia and sporosori in epidermal root cells. Confocal microscopy was useful in differentiating real plasmodial structures from the false positives caused by lactophenol blue staining of such a large mass of root hairs. Pieces of roots were mounted in demineralized water and were analyzed using a Zeiss LSM 5 exciter (Carl Zeiss AG, Jena, Germany) confocal microscope, with a 488 nm excitation laser and a 505 nm barrier filter. In order to confirm the identity of the observed structures as those of *P. betae* a specific PCR was carried out. First, the total DNA of unstained parts of roots showing *P. betae* structures was extracted using a FastDNA Kit (MP Biomedicals, Solon, OH, USA), following the manufacturer's protocol. A *Polymyxa*-specific PCR was performed using the Psp1 (5'-TAGACGCAGGTCATCAACCT-3') and Psp2rev (5'-AGGGCTCTCGAAAGCGCAA-3') primers designed by Legrève *et al.* (2003).

2.2.3 Results and discussion

None of the twenty syringes showed external contamination by others microorganisms, such as fungi or bacteria. Plasmodia and sporosori were detected using lactophenol blue staining. Their morphology was similar to that observed for *P. betae* on *B. vulgaris* (fig 2.7, b. and c.). Plasmodia, sporosori, and meronts (i.e. individual plasmodia at an early stage in resting spore formation) were observed with $DiOC_6(3)$ staining (Fig. 2.6), which was useful for observing the protist structures in the numerous infected root hair cells. Infection by *P. betae* was detected in 10 out of the 20 inoculated hairy roots in which 20-80% of the cells were infected, including cells in the root hairs, epidermis, and cortex. The positive results with the specific PCR strengthen the argument that the observed structures were those of *P. betae* (data not shown). Zoosporangia were not visible, but the great number of *P. betae* structures in

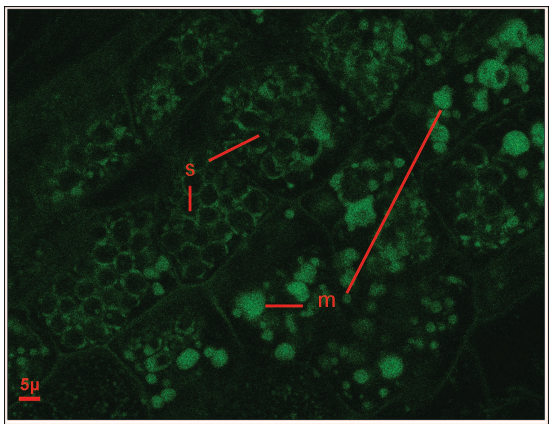

Fig. 2.6 : Confocal microscopy of a single section of the apex of the hairy root of the sugar beet *Beta vulgaris* containing sporosori (s) and meronts (m) of the plasmodiophorid *Polymyxa betae*. Staining with $DiOC_6(3)$.

parts of the hairy roots produced after the first inoculation confirmed that secondary infection had occurred.

Mugnier (1987) reported efforts to develop a dual culture of *P. betae* on sugar beet hairy roots. His fig. 5 (p. 541, Mugnier (1987)) illustrating *P. betae* infection in a hairy root in fact shows a typical structure of *Phialophora sp.*, a soil-borne fungal parasite of roots. Since no serological or molecular test was carried out in that study to prove the presence of *P. betae* infection in hairy roots, Mugnier's conclusions on the infection of hairy roots by *P. betae* can be challenged. Our work confirms the presence of typical structures of *P. betae* observed by optical and confocal microscopy, and backed up by molecular analysis. This is the first system for cultivation of plasmodiophorids developed in liquid media that enables secondary infection by zoospores. Moreover, roots cultivated in these systems can be transplanted in new syringes with fresh medium. With this new *in vitro* system, it will be possible to produce zoospores and sporosori *en masse*. The system will be useful for multiplying and conserving strains and producing an axenic inoculum of the protist, thus contributing to a greater understanding of this plasmodiophorid.

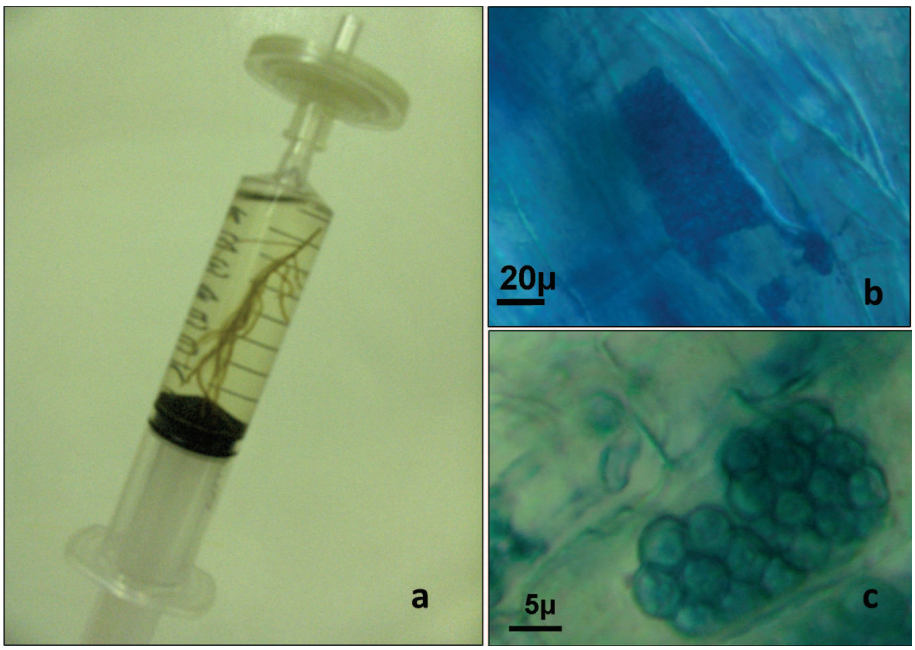

Fig. 2.7 : a. Culture system of hairy roots of the sugar beet *Beta vulgaris* in a 20-mL syringe after 10 weeks of incubation. b. Plasmodia of *Polymyxa betae* stained with lactophenol blue in epidermal cells of *Beta vulgaris* hairy roots. c. Sporosori of *P. betae* stained with lactophenol blue in epidermal cells of the *Beta vulgaris* hairy roots.

2.3 Chapter conclusion

In this chapter, we investigated two new study models for the protist *P. betae*.

The first model aimed to identify compatible interactions between *P. betae* and *A. thaliana*. Various ecotypes of *A. thaliana* were challenged with an aviruliferous isolate of *P. betae*. Compatible interactions were obtained but the infection level was lower in comparison with sugar beet and the phenotype of the protist in *A. thaliana* differed from those in sugar beet. Therefore, in spite of the fact that *A. thaliana* is a plant providing numerous tools to study its transcriptome, we decided to not continue the use of this model in the next part of this thesis. Indeed, a changing phenotype could be the sign of other significative changes in the transcriptomes of the plant or the protist. However, *A. thaliana*-*P. betae* model is a valuable tool for other applications such as a deeper sequencing of *P. betae* or transcriptome comparative analysis with those made on beet.

The second model presented in this chapter aimed to develop a method allowing the production of great quantities of zoospores free of other soil or roots contaminants. A dual culture of *P. betae* in sugar beet hairy roots was obtained. This culture allows a "microorganism-free" inoculum of *P. betae* but the developed model, at this stage of development, did not allow a mass production of zoospores. For the subsequent experiments of these thesis, that required a large number of zoospores, we did not use this system. Nevertheless, this system allows the production of "pure" zoospores, enabling future sequencing of *P. betae* genes. Moreover, it could be valuable in studies with viruliferous *P. betae* and for long-term storage and multiplication of pure isolates *in vitro*.

CHAPTER 3

STUDYING INTERACTOME THROUGH GENE DIFFERENTIAL EXPRESSION ANALYSIS

Opening comments

The transcriptome analysis is an approach which provides a snapshot of the needs and activities of a target organism. Up to now, the most effective techniques to analyze transcriptome are the use of microarrays, the Serial Analysis of Gene Expression (SAGE) or RNA-seq. However, these techniques require an extended knowledge about the considered genome. This is neither the case of sugar beet nor *Polymyxa betae*. The suppression subtractive hybridization (SSH) is another transcript analysis technique which allows a qualitative differential transcript analysis without requiring genome knowledge of the concerned organisms. This technique was applied in an experiment developed to improve knowledge about the sugar beet-*P.betae* interaction. This experiment enabled to highlight genes of sugar beet involved in this interaction and to learn more about the parasite genes. Some genes were selected from each protagonist and their expression was followed along the successive steps of *P. betae* infection in sugar beet.

3.1 Molecular interactions between sugar beet and *Polymyxa betae* along the protist life cycle

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3.1.1 Introduction

Rhizomania is one of the most important diseases of sugar beet. The causal agent of the disease, the *Beet necrotic yellow vein virus* (BNYVV) is a soilborne virus transmitted by *Polymyxa betae*. This protist belongs to the family of Plasmiodiophorids, the phylum *Cercozoa* and the class *Phytomyxea* (Adl *et al.*, 2005). Some members of this family cause direct lesions on infected plants such as powdery scab of potato caused by *Spongospora subterranea* or clubroot disease of crucifers caused by *Plasmodiophora brassicae*. Three members such as *Polymyxa graminis*, *P. betae* and *S. subterranea* are involved in the transmission of phytoviruses on major crops (Dieryck *et al.*, 2011; Tamada & Baba, 1973). Due to their obligate endoparasite nature, the molecular biology of plasmodiophorids remains largely unknown because molecular investigations are hampered by various obstacles. First, it is impossible to culture them alone axenically. Only dual cultures of these organisms within hairy roots from one of their hosts were developed (Desoignies & Legrève, 2011; Qu & Christ, 2007). Secondly, their intracellular nature and the fact that most of their host plants are not fully sequenced make difficult the segregation of the host and parasites genomes or transcriptomes. The development of new model studies on model plant species such as described in Desoignies *et al.* (2011) could also help to improve knowledge on this genus and group. With regard to these difficulties, the known coding sequences of plasmodiophorids are limited to the actin and ubiquitin genes for some of them (Archibald & Keeling, 2004; Bulman *et al.*, 2001),

the trehalose-6-phosphate synthase gene from *Plasmodiophora brassicae* (Brodmann *et al.*, 2002) and 76 *P. brassicae* gene sequences obtained following suppression subtractive hybridization (SSH) between cDNA banks from *P. brassicae*-infected and uninfected *Arabidopsis* tissue (Bulman *et al.*, 2006). Few other sequences are known as parts of the ribosomal sequences and the internal transcribed spacer (ITS) regions, which were used to develop broad spectrum or specific *Polymyxa* detection tools (Legrève *et al.*, 2002; Vaianopoulos *et al.*, 2007; Ward & Adams, 1998; Ward *et al.*, 2005). Studies of molecular interactions between plasmodiophorids and their host are relatively poor: only *Plasmodiophora brassicae* is relatively documented, principally thanks to its host range containing plant model organisms, such as *A. thaliana* and *Brassica napus*, which are completely sequenced. In this pathosystem, events as gall formation (Malinowski *et al.*, 2012), role of defense ways such as ethylene signaling pathway (Knaust & Ludwig-Müller, 2012) or global gene expression of the host during the infection (Agarwal *et al.*, 2011) have been recently studied. Up to now, the research on interactions between the other economically significant plasmodiophorids (*Spongospora subterranea*, *Polymyxa graminis* and *P. betae*) and their hosts is restricted to some investigations using dual cultures (Bulman *et al.*, 2011; Qu & Christ, 2007) or differential transcripts analysis (McGrann *et al.*, 2007, 2009*b*). In order to better understand the interactions between *P. betae* and sugar beet, the strategy developed by Bulman *et al.* (2006) and Sundelin *et al.* (2011) for *P. brassicae* was applied, in this study, to the *P. betae* - *Beta vulgaris* interaction. The aim was to highlight some genes expressed by the protist and its host during its life cycle, using suppression subtractive hybridization (SSH) (Diatchenko *et al.*, 1996). Expressed sequence tags (ESTs) of *B. vulgaris* and *P. betae* during the primary infection period (within 24 h of inoculation) and later in the cycle of the protist (5 and 15 days post inoculation) were first identified. The dynamics of selected *P. betae* and *B. vulgaris* genes expression along the life cycle of the protist was then assessed by a quantitative RT-PCR analysis. In addition to provide information on biological processes involved in the interaction protist-plant such as the induction of plant defense ways, 76 ESTs of *P. betae* were obtained.

3.1.2 Materials and methods

***P. betae* inoculum** Zoospores of *P. betae* were obtained by culturing a monosporosorous and aviruliferous isolate (A_{26-41}) on sugar beet var. Cadyx (SES Vanderhave, Tienen, Belgium) in an automatic immersion system (AIS) (Legrève *et al.*, 1998) as described by Desoignies & Legrève (2011).

Plant growth and *P. betae* inoculation Seeds of sugar beet var. Cadyx were dissected in order to separate the embryo from the pericarp. The embryos were disinfected for 15 min in 2.5% NaClO and then rinsed for 2 h in demineralised sterile water. After 2 days of pre-germination in the dark at room temperature, the seedlings were transferred on quartz in tubes placed in an AIS. The plants were irrigated with Hoagland solution (pH 7.2) for 1 h every 3 h in an environmental cabinet with a photoperiod of 14 h and at 25°C - 20°C (day-night temperature). After 15 days of growth, 10 plants were each inoculated with a total of 30,000 *P. betae* zoospores and 10 other plants with a total of 10,000 zoospores. Twenty plants were left uninoculated as a negative control and driver for SSH. The zoospores quantities of 30,000 and 10,000 were chosen according to the time left between the inoculation and the analysis. For the plants harvested during the first day post-inoculation, 30,000 zoospores were inoculated in order to maximize the detection of *P. betae* and sugar beet genes involved in their interaction. For the plants harvested five and fifteen days after inoculation, less zoospores (10,000) were inoculated in order to avoid a "saturation" of the host cells by *P. betae* and the early formation of sporosori. The plants were then grown in the same conditions as described above, but with an irrigation of six hours every twelve hours to stimulate *P. betae* development. Plant root systems were harvested at four times after the inoculation, representing successive stages of life of the protist. Five root systems inoculated with 30,000 zoospores were harvested 3 h after inoculation, corresponding to the entry of zoospores into host cells, and another five were harvested 24 h after inoculation, at the beginning of plasmodial stage. Five root systems inoculated with 10,000 zoospores were harvested 5 days after inoculation, at the plasmodial stage, and another five were harvested 15 days after inoculation, during the sporangial and sporogenic phases. At each

harvest time, five uninoculated plants were also harvested. Some roots of the different samples were boiled for 2 min in cotton blue lactophenol in order to assess the *P. betae* structures present at each sampling by light microscopy.

RNA preparation The total RNA of the each root sample was extracted using Trizol reagent (Invitrogen, Carlsbad, USA) and then purified with the PureLink RNA minikit (Invitrogen), following the manufacturer's instructions. DNA was removed from RNA extracts by DNase1 (Roche, Basel, Switzerland) digestion. Then, the mRNAs were isolated from the different samples using the PolyATtract-mRNA isolation kit (Promega, Madison, USA). The mRNAs were then pooled as following. The mRNAs corresponding to the inoculated plants harvested 3 h after inoculation were pooled with those obtained from samples harvested 24 h after inoculation, the mRNAs from the negative control roots harvested at these two times were also pooled. The mRNAs corresponding to the inoculated plants harvested 5 days and 15 days after inoculation were also grouped, as the mRNAs from roots of sugar beet non-inoculated with *P. betae*, harvested at these two other times.

Suppression subtractive hybridization Two "forward" subtractions were performed using the PCR-Select cDNA Subtraction Kit (Clontech, Mountain View, USA) in order to obtain *P. betae* and *B. vulgaris* sequences. The subtractions used mRNAs from *P. betae*-infected sugar beet at different times as testers and the mRNAs from healthy sugar beets at corresponding times as drivers of SSH. The subtraction "infection" (pool of 3 and 24 h-infected plants subtracted with healthy plants of the same age) was conducted to identify genes corresponding to the response of sugar beet expressed during zoospore penetration and the first hours of the protist into the sugar beet root cell. The second subtraction was conducted to identify genes expressed in response to the endoparasitic phase of *P. betae* life cycle, during the growth (5 days after inoculation), multiplication and survival (15 days after inoculation) phases. PCR fragments generated by the two SSHs were cloned in the pGEM-T vector (Promega). For each subtraction, 450 clones were randomly selected and sequenced. The sequencing was performed by

Macrogen Inc. (Seoul, Korea) using the universal primers M13F and M13R .

Bioinformatic analysis The quality of the DNA sequences obtained from SSH clones was manually checked, taking Phred quality scores into account (Richterich, 1998). The plasmid sequences were manually removed from raw sequences. Overlapping sequences were assembled using the CAP 3 Sequence Assembly Program. Segregation between sugar beet and *P. betae* sequences was done using the MIPS EST classification server (Emmersen *et al.*, 2007). Two databanks were created to train the model. The first databank contained 100 sequences of *B. vulgaris* randomly chosen from the NCBI databank. The second databank was constructed from randomly chosen sequences of plasmodiophorids (*P. betae*, *Plasmodiophora brassicae*) and *Cercozoa* (*Bigelowiella natans*, *Gymnophrys cometa* and *Paracercomonas marina*). The data mining and functional analysis of the original sequences obtained in our study were done using the Blast2Go research tool (Conesa *et al.*, 2005). Sequences were mapped with their corresponding gene ontologies (GO) terms.

Gene expression profiling

Bioassay Time series gene expression profiles of *Polymyxa betae* and sugar beet genes putatively involved in the interaction between both organisms were analyzed in order to assess their expression levels along the distinct stages of the *P. betae* life cycle. For this purpose, a novel bioassay was performed. Sugar beets were grown in individual glass culture tubes as described in Desoignies *et al.* (2011). Fifteen days after germination, a half of plants were inoculated with *P. betae* zoospores (5,000 zoospores/plant). The zoospores were produced as described above. Sets of 4 *P. betae* inoculated (Pb+) and 4 non-inoculated plants (Pb-) were harvested 3 hours, 24 hours, 5 days and 15 days after *P. betae* inoculation. Each collected root was sampled, deep frozen and stored at -80°C before the mRNAs extraction (see above).

Gene selection and primer design From the 76 ESTs from *P. betae* identified in the first part of this study, nine sequences were selected on

the basis of their putative role in the *P. betae* biology or in the interaction with sugar beet. The four *P. betae* housekeeping genes candidates were the beta-tubulin, the actin and histone, three usual housekeeping genes (Hruz *et al.*, 2011), and glutathione-S-transferase. The later was selected based on the work of Kingsnorth *et al.* (2003) reporting the good correlation between protein production and *P. betae* quantity the first fifteen days of infection. Three other *P. betae* genes putatively involved in infection and parasitic processes were selected. The expression of a gene putatively encoding for polysaccharide deacetylase (PbPode), protein enabling the digestion of polysaccharides such as cellulose as well as two genes suspected to play a role in the infection phase, the profilin (PbPro) and the von Willebrand factor domain containing protein (PbvWf), were followed. The expression of two genes putatively involved in signaling processes were also profiled: a G coupled receptor protein (PbGP) and a galactose-binding lectin. For the sugar beet gene expression profiling during the interaction with *P. betae*, three *B. vulgaris* constitutive genes were selected as housekeeping gene for the normalization of qRT-PCR: β-tubulin, *GAPDH* and Histone 3a. The expression of 11 other sugar beet genes were profiled. Four genes putatively encoding for PR-proteins: a cystein-rich protein (BvCys), likely a gamma-thionin or defensin, from the PR-12 or -13 family, the *Beta vulgaris* protease inhibitor (BvPin) (PR-6 family) and two proteins from the PR-10 family (BvPR10 and BvRip) (Van Loon *et al.*, 2006). Two genes probably involved in defense response and apoptosis (jacalin, BvJac, and proteasome subunit, BvProt) were also followed. Other genes, encoding putatively for more specific defense proteins, were selected to be followed: a translationally controlled tumour protein (BvTum), a RNA-dependent-RNA polymerase (BvRpolR), a remorin (BvRem), and a salt-induced protein (BvSI), protein of stress response. Endly, the gene coding for a protein with an unknown function, jasmonate-induced protein (BvJI), but induced by jasmonate and thus related to defense ways of the plant was also tested. The primer pairs specific to the selected sequences were designed using the primer-BLAST tool of NCBI, targeting regions between 150 and 200 bp, and an annealing temperature of 62°C. Gene names, putatively associated functions and primers are listed in tables 3.1 and 3.2. In order to confirm that the nine targeted *P. betae* sequences belong to *P. betae*, PCR using primer pairs designed to amplify

these sequences were performed using DNA extracts from healthy and *P. betae*-infected sugar beets under the same PCR conditions as detailed in the qRT-PCR described below.

Table 3.1: Primers used for *P. betae* genes. The primers were designed with Primer-BLAST tool (NCBI). Use: Housekeeping gene (HKG) or tested gene (T). vWf. von Willebrand factor.

Gene name	Gene product	Use	Primer name	Primer sequence
PbA	actin	HKG	PbAFor	5'-CGGAAGAGCATCCCGTTTTGCTG-3'
			PbARev	5'-ATCCAAACGGAGAATGGCGTTGGGG-3'
PbBT	beta-tubulin	HKG	PbBTFor	5'-CACTGGTACACCGGTGAGGGCATG-3'
			PbBTRev	5'-TGTTGGACTGGCCCAGGGATTGGG-3'
PbGlec	galactose-binding lectin	T	PbGlecFor	5'-AGCACCGAGCAACAACGGCCA-3'
			PbGlecRev	5'- TGCCGAGTTTGGGGGCGTGAA-3'
PbGP	G-protein coupled receptor	T	PbGPFor	5'-TCGACTCGGGTGGTTGGCGTCG-3'
			PbGPRev	5'-TCGACTCGGGTGGTTGGCGTCG-3'
PbGST	glutathione-S-transferase	HKG	PbGSTFor	5'-TCGCGACCGGCCCGAACAAG-3'
			PbGSTRev	5'-GGGCGAGTTCCGGAAGCCGGC-3'
PbHis	histone	HKG	PbHisFor	5'-GGCGCTGGTCGCGGGTAAAGC-3'
			PbHisRev	5'-CGGCGTTGCCGGCCAATTCAA-3'
PbPro	profilin	T	PbProFor	5'-GGATGGGCGGCCTGGAACGA-3'
			PbProRev	5'-GACAGCGCTGGAGTCGCCGAG-3'
PbPode	polysaccharide deacetylase	T	PbPodeFor	5'-CGTACGGTGGGACGACCGGC-3'
			PbPodeRev	5'-TCACCGTTGCCGTTGTGGTGGA-3'
PbvWf	vWf domain containing protein	T	PbvWfFor	5'-TCGGAGCGAGAAGCGCGAGAT-3'
			PbvWfRev	5'-CCGGGTTGATGCGTGGCCCTA-3'

qRT-PCR The gene expression profile of each gene was assessed by qRT-PCR. The concentration of RNA extracts was adjusted to 2 ng per ml before the reverse transcription step. cDNA synthesis was performed in two steps: first, a mix of 1 μl reverse primer, 8.5 μl of DEPC water and 1 μl RNA was incubated for 10 min at 65°C. Secondly, a reaction mixture of 4 μl of M-MLV RT buffer, 0.25 μl of M-MLV reverse transcriptase (200 U/ μl) (Promega), 2 μl of dNTP (20 nmol) and 3.5 μl of DEPC water were added to the first reaction mix and incubated at 42°C for 60 min. qPCR was conducted using synthesized cDNA as template DNA. SYBR-Green chemistry was used in this study for the quantitation of DNA products. The reaction components for each sample were 10 μl iQ SYBR Green Supermix, 0.4 μl of each primer (20 pmol), 7.2 μl of DEPC water and 2 μl of the cDNA. Amplification reaction was performed using the CFX 96 Real-Time System of Biorad, as follows: first, the denaturation step at 95°C for 3 min, then 40 cycles of 30s at 95°C, 30s at 62°C and 30 s at 72°C, and finally a melting curve was established. Two duplicates of the qPCR were made for each sample, standard and control and the mean of the two duplicates was taken in account. Standards were obtained by conducting RT-PCR on *P. betae* infected sugar beet extract, under the same conditions than qRT-PCR and by making serial dilutions of the PCR products, in order to establish a standard curve. Only standard curves with an efficience greater than 90% were taken in account. The gene expression profiling and quality analysis were done using the Biorad CFX manager 2.1 taking account of the MIQE recommendations (Bustin *et al.*, 2009).

Table 3.2: Primers used for *B. vulgaris* genes. The primers were designed with Primer-BLAST tool (NCBI). Use: Housekeeping gene (HKG) or tested gene (T)

Gene name	Gene product	Use	Primer name	Primer sequence
BvBT	beta-tubulin	HKG	BvBTFor	5'-TGCTACCCTCTCCGTCCATCAGCTT-3'
			BvBTRev	5'-ACCAGGGAACATCAGGCAGCAGG-3'
BvCys	cystein-rich protein	T	BvCysFor	5'-CGCGGTTTCCGCCGTCGTTG-3'
			BvCysRev	5'-GGGTTCGCCACTGCCTTTATGCCTT-3'
BvGAPDH	glyceraldehyde phosphate dehydrogenase	HKG	BvGAPDHFor	5'-CGACCACTTCGCAAAGCTCGTGT-3'
			BvGAPDHRev	5'-AACGCTGAATGAATGCACGCCGA-3'
BvHis	histone 3a	HKG	BvHisFor	5'-AGCCACGCTGTGTTGGCCCT-3'
			BvHisRev	5'-AGCACGTTCACCCCTGATTCTGCG-3'
BvJac	jacalin-like domain containing protein	T	BvJacFor	5'-CGCGTTTCAACTTGTTGATGGTGAGC-3'
			BvJacRev	5'-GGCGAGCCACTGCTACCGACC-3'
BvJI	jasmonate-induced protein	T	BvJIFor	5'-AACGCTGCCTGGCTCGTTGCT-3'
			BvJIRev	5'-TTGGCCACCCCCCGGTTCTCA-3'
BvPin	protease inhibitor	T	BvPinFor	5'-TCCAAGCTTATGCATCCAACGGCG-3'
			BvPinRev	5'-CGGGCCACGTATTCTTTCCTTGGCA-3'
BvPR10	PR-10 protein	T	BvPR10For	5'-AGAAGTTGGAGGGGCAAGTAGAGC-3'
			BvPR10Rev	5'-TCTCCTGACGCTACCCACGCA-3'
BvProt	proteasome subunit beta type 6	T	BvProtFor	5'-CCTTGCCATTGCTCGAGATGGTGCT-3'
			BvProtRev	5'-TGCCATTGGCTCAGGGCTTGCAG-3'
BvRem	remorin	T	BvRemFor	5'-GCTTGGGATGAAGCAGAGCGTGC-3'
			BvRemRev	5'-CGAGCTGCAGCGAGTTTATTGGCG-3'
BvRip	ripening-related protein	T	BvRipFor	5'-AGGGGGACTGGAACAAAGTTGGTGG-3'
			BvRipRev	5'-CCTGCACCTTGGAGGTCAAGCTCT-3'
BvRpolR	RNA-dependent RNA polymerase	T	BvRpolRFor	5'-TGGCAAAGTGGCCCTGCGGC-3'
			BvRpolRRev	5'-GGGTCCCAGCTGATTACCTTGCTCG-3'
BvSI	salt-induced protein	T	BvSIFor	5'-TGCGCTGAATGGAGGTGGAAAAGT-3'
			BvSIRev	5'-TGTGTTCAGGCGCATTCACCTTCG-3'
BvTum	translationally controlled tumour protein	T	BvTumFor	5'-AGCTCCTGACTGGAAAGTTGGACGC-3'
			BvTumRev	5'-TCGTCACCCATGCTCTCGCCCA-3'

3.1.3 Results

Microscope observations No structure of *Polymyxa betae* was observed in cotton blue lactophenol stained samples of sugar beet roots collected 3 h and 24 h following the inoculation. In the roots sampled 5 days after the inoculation, some typical plasmodia of the protist were visible in the roots. In the root fragments stained 15 days after inoculation, typical structures of each stage of the life cycle of *P. betae*, plasmodia, zoosporangia (multiplication phase) and sporosori (survival phase), were visible. Plasmodia differentiating into zoosporangia and sporosori were also observed.

ESTs analysis A total of 186 unisequences were obtained from the first subtraction (3-24 hours post-infection) aiming to identify ESTs expressed during th *P. betae* infection phase and 233 unisequences from the second subtraction (5 and 15 days post-infection) performed to identify ESTs from multiplication and survival phases. A total of 61 sequences were identified as *P. betae* sequences in the first case and 140 in the second. Matches with known proteins or functions were obtained for 22 sequences during the infection phase and for 54 sequences during the rest of the cycle. The ESTs ranged from 137 to 1105 bp, with a mean of 530 bp. The sensitivity of the MIPS EST3 model on the test set was 97 %. The results are showed in table 3.3. From the 76 obtained *P. betae* ESTs, 54 predicted peptidic sequences matched with a protein of known function, 11 contained conserved domains identified by pFam, and 11 matched with proteins of unknown function. Six sequences matched with ribosomal proteins. Within these ribosomal sequences, two matched with protist sequences: ribosomal proteins of *Theilera parva* and *Plasmodiophora brassicae*. One unisequence matched (95%) with a known *P. betae* sequence: the glutathione-S-transferase identified by Mutasa-Göttgens *et al.* (2000). In genes coding for transcription, the ESTs referred as 'PbRNApol' and 'PbRNApol2' were quasi similar (after alignment with ClustalW, only one mismatch on 855 bases) and were expressed from the same gene during both periods of analysis (3-24 h, and 5-15 days). Three of the four stress-associated genes (table 3.3) had the putative same functions as those reported in the study performed on *Plasmodiophora brassicae* by Bulman *et al.* (2006): a heat-shock protein 'PbHSP20', a

chaperone, 'PbCpn' and a glutathione-S-transferase 'PbGST'. In addition, an EST coding for the cytochrome P450, 'PbCytP', with a known detoxifying function was identified. These four last ESTs are in the pool of sequences expressed 5 and 15 days after inoculation.

For sugar beet, 126 ESTs were obtained for the pool 3-24h and 92 for 5 and 15 days. After bioinformatics analysis, respectively 77 and 43 EST were mapped with known gene ontologies (GO). Sequences with putative interaction or defense function are presented in table 3.4. For the pool 3 and 24h, 17 sequences (about 22%) were mapped with function of defense or putative interaction. For 5 and 15 days, nine sequences (21%) were mapped with the same kind of function. Two sequences are common in the two pools: a pathogenesis protein from the family 10 and a jacalin-like domain containing protein.

Gene expression profiling

P. betae

Bands corresponding to the expected molecular weights for the nine targeted DNA sequences from *P. betae* (PbA, PbBT, PbGlec, PbGP, PbGST, PbHis, PbPro, PbPode and PbvWf) were observed on the electrophoresis gel under UV light, after PCR amplification using DNA extracts from *P. betae* infected sugar beet plants. No signal was obtained using DNA extracts from *P. betae*-free sugar beet. This result confirms that the targeted ESTs belong to *P. betae*.

In order to test the relevance of four housekeeping genes for the data normalization in qRT-PCR, linear regression analysis of the relative expression progress over time was done for each tested gene. The genes showing similar slopes, indicating a common increase of expression related to multiplication of *P. betae* and thus *P. betae* RNA, were chosen as housekeeping genes. Expression profiles over time of candidates housekeeping genes are shown on the fig. 3.1.

Table 3.3: ESTs from *P. betae* obtained in this study. Length is in bp.

Name	Accession	Time	Length	Protein information	e-value	Organism
Ribosomal proteins						
PbL23	FR847928	5-15d	553	ribosomal L23	1,00E-47	*Gallus gallus*
PbL16	FR847933	5-15d	700	ribosomal $L16_L10$	1,00E-83	*Theileria parva*
PbS17	FR847939	5-15d	582	ribosomal S17e	2,00E-44	*Ostreococcus tauri*
PbL1	FR847936	5-15d	574	ribosomal L1	4,00E-42	*Trichoplax adhaerens*
PbL37ae	FR847138	3-24h	216	ribosomal L37ae	4,00E-11	*Arabidopsis thaliana*
PbL7ae	FR847139	3-24h	450	ribosomal L7ae	1,00E-55	*Plasmodiophora brassicae*
Transcription						
PbRNApol2	FR847925	5-15d	855	RNA polymerase	3,00E-03	*Physcomitrella patens*
PbZf2	FR847897	5-15d	317	zinc finger	4,00E-11	*Candida glabrata*
PbTub	FR847134	3-24h	561	Tub superfamily	5,00E-13	*Vitis vinifera*
PbZfH	FR847135	3-24h	407	Zf-HIT	1,00E-05	*Glossina morsitans*
PbRNAPol	FR847136	3-24h	857	RNA polymerase	2,00E-04	*Antonospora locustae*
PbNot	FR847137	3-24h	666	Not 1 transcription factor	5,00E-54	*Micromonas* sp.
Stress associated						
PbHSP20	FR847905	5-15d	438	HSP20	2,00E-21	*Blastocystis hominis*
PbCpn	FR847917	5-15d	239	chaperonin cpn10	8,00E-11	*Plasmodium knowlesi*
PbCytP	FR847941	5-15d	829	cyt p450	2,00E-25	*Pediculus humanus*
PbGST	FR847943	5-15d	685	glutathione-s-transferase	9,00E-96	*Polymyxa betae*
Metabolism						
Pbchit	FR847942	5-15d	378	chitin synthase	6,00E-13	*Leptosphaeria maculans*
Pbnuc	FR847918	5-15d	215	nuclease homologue	5,00E-03	*Drosophila melanogaster*
PbHis	FR847938	5-15d	249	core histone H2A	6,00E-20	*Ailuropoda melanoleuca*
PbHis2	FR847889	5-15d	602	histone	9,00E-51	*Adineta vaga*
PbH3	FR847900	5-15d	596	histone H3	4,00E-67	*Xenopus tropicalis*
PbPolsy	FR847906	5-15d	388	polysaccharide synthase	2,00E-08	*Coprinopsis cinerea*
PbPode	FR847124	3-24h	528	polysaccharide deacetylase	5,00E-10	*Plasmodiophora brassicae*
PbPode2	FR847909	5-15d	688	polysaccharide deacetylase	6,00E-04	*Plasmodiophora brassicae*
PbPode3	FR847926	5-15d	713	polysaccharide deacetylase	7,00E-14	*Streptomyces viridochromogenes*
PbHD	FR847921	5-15d	260	haloacid dehalogenase like hydrolase	2,00E-21	*Phytophthora infestans*
Signaling						
PbGP	FR847893	5-15d	610	G-protein alpha subunit	2,00E-21	*Polysphondylium pallidum*

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Table 3.3: ESTs from *P. betae* obtained in this study. Length is in bp.

Name	Accession	Time	Length	Protein information	e-value	Organism
PbGlec2	FR847891	5-15d	544	galactose binding lectin	7,00E-04	*Ostreococcus lucimarinus*
PbGlec	FR847133	3-24h	469	galactose-binding lectin	1,80E-04	-
Pbef1	FR847922	5-15d	430	SPRY Domain	5,00E-05	*Phytophthora infestans*
Pbef2	FR847907	5-15d	840	cystein-rich secretory protein	9,00E-10	*Stigmatella aurantiaca*
Protein Fate						
PbPCI	FR847919	5-15d	607	PCI domain	1,00E-46	*Nematostella vectensins*
Pbkin	FR847896	5-15d	303	protein Kinase	6,00E-11	*Phytophthora infestans*
PbEF	FR847932	5-15d	594	eukaryotic elongation factor 5A hypusine	3,00E-35	*Salmo salar*
PbSC	FR847929	5-15d	561	serine carboxypeptidase	1,00E-16	*Equus caballus*
PbPEPM16	FR847130	3-24h	465	peptidase M16C	5,00E-12	*Schizosaccharomyces japonicus*
Transport						
PbHATPase	FR847940	5-15d	785	H+ATP-ase	3,00E-13	*Trypanosoma cruzi*
PbCl	FR847937	5-15d	372	clathrin adaptor complex small chain	3,00E-27	*Oryza sativa*
PbChol	FR847144	3-24h	250	plasma-membrane choline transporter	9,00E-03	*Thalassiosira pseudonana*
Others						
PbPro	FR847935	5-15d	515	profilin	8,00E-05	*Oikopleura dioica*
PbA	FR847904	5-15d	650	actin	1,00E-82	*Spongospora subterranea*
PbBT	FR847924	5-15d	507	beta tubulin	5,00E-68	*Plasmodiophora brassicae*
PbKaz	FR847125	3-24h	466	$kazal_1$	3,00E-04	*Cryptosporidium hominis*
PbPPa	FR847127	3-24h	464	phosphopantetheine attachment site	2,00E-24	*Picea sitchensis*
PbMtr	FR847126	3-24h	762	methyltransferase D12	2,00E-09	*Janthinobacterium* sp. Marseille
PbCub	FR847128	3-24h	137	Cu-bind like	1,00E-10	*Cucumis sativus*
PbZf	FR847129	3-24h	790	zinc finger protein 36	9,00E-14	*Xenopus laevi*
Pbtr	FR847898	5-15d	773	trichohyalin	4,00E-07	*Toxoplasma gondii*
PbCytC	FR847131	3-24h	522	cytochrome c ox. VI a	3,00E-09	*Ricinus communis*
Pbubox	FR847132	3-24h	388	NADH-ubiquinone oxidoreductase	7,00E-06	*Micromonas pusilla*
PbPPbind	FR847927	5-15d	458	PP-binding prot	7,00E-23	*Picea sitchensis*
PbTyr1	FR847908	5-15d	352	tyrosinase	7,00E-09	*Sepia officinalis*
PbTyr3	FR847931	5-15d	323	tyrosinase	6,00E-04	*Rhizobium etli*
Unknown function with predicted domain						
PbUN7	FR847910	5-15d	422	ankyrin repeat	7,00E-07	*Trichomonas vaginalis*
PbUN8	FR847911	5-15d	463	reeler domain	3,00E-03	*Danio rerio*

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Table 3.3: ESTs from *P. betae* obtained in this study. Length is in bp.

Name	Accession	Time	Length	Protein information	e-value	Organism
PbPTr	FR847916	5-15d	665	PT-repeat containing protein	1,00E-03	*Burkholderia ambifaria*
PbUN10	FR847899	5-15d	513	ankyrin repeat containing protein	5,00E-05	*Debaryomyces hansenii*
PbUN11	FR847915	5-15d	605	associated lipoprotein	0,062	*Borrelia burgdorferi*
PbvwF	FR847912	5-15d	488	von Willebrand factor domain	7,00E-06	*Sclerotinia sclerotiorum*
PbUN13	FR847930	5-15d	352	acyl co A binding protéin	1,00E-18	*Micromonas pusilla*
Pbreel	FR847894	5-15d	523	similar to reeler domain	3,00E-08	-
PbPPRr	FR847895	5-15d	517	PPR repeat containig protein	1,00E-14	*Selaginella moellendorffii*
PbUN18	FR847914	5-15d	607	zinc finger domain	4,00E-22	Ca*enorhabditis remanei*
PbUN3	FR847143	3-24h	385	DUF1253 family	8,00E-03	-
Unknown function						
PbUN19	FR847903	5-15d	713	unknown	3,00E-104	*Paramecium tetraurelia*
PnUN1	FR847140	3-24h	681	unknown	2,00E-03	*Plasmodium falciparum*
PBUN2	FR847141	3-24h	514	unknown	8,00E-04	*Polysphondylium pallidum*
PbUN4	FR847142	3-24h	617	unknown	3,00E-05	B*ranchiostoma floridae*
PbUN5	FR847145	3-24h	661	unknown	2,00E-11	*Blastocystis hominis*
PbUN6	FR847913	5-15d	266	unknown	3,00E-08	*Schizophyllum commune*
PbUN9	FR847923	5-15d	676	unknown	2,00E-03	*Plasmodium falciparum*
PbUN14	FR847888	5-15d	864	unknown	2,00E-09	*Lentisphaera araneosa*
PbUN15	FR847890	5-15d	401	unknown	1,00E-03	*Polysphondylium pallidum*
PbUN16	FR847901	5-15d	1105	unknown	9,00E-12	*Blastocystis hominis*
PbUN17	FR847902	5-15d	407	unknown	1,00E-02	*Physcomitrella patens*

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Table 3.4: *B. vulgaris* ESTs related to stress or defense response obtained in this study. Accession numbers are not yet allocated. Length is in bp.

Name	Length	Protein information	e-value	Biological process
3h-24h post infection				
BvCys	753	cystein-rich protein (gamma-thionin)	1,4 e-08	defense response, cell killing
BvHom	479	homeostasis protein	3,6 e-44	cell redox homeostasis
BvHSP	512	heat shock protein	5 e-24	response to stress
BvJac	676	jacalin like domain containing protein	2 e-15	defense response, apoptosis
BvJI	436	jasmonate-Induced protein	3,9 e-05	unknown function
BvLtp	193	lipid transfer protein	2,6 e-19	defense response
BvMip	468	protein from MIP/PIP subfamily	2,1 e-12	water transport
BvPAL	430	phenylalanine ammonia lyase	7,4 e-34	defense response
BvPin	576	protease inhibitor	1,9 e-13	defense response
BvPR10	364	pathogenesis related protein, family 10	2 e-15	putative RNAse activity
BvRamp	463	ribosome associated membrane protein	2,3 e-12	response to oxidative stress
BvRem	609	remorin	3,9 e-17	putative action on plasmodesmata
BvRip	823	pathogenesis related protein, family 10	1,9 e-34	putative RNAse activity, ripening-related protein
BvRpolR	582	RNA polymerase RNA-dependent	2 e-21	gene silencing
BvSI	676	salt-induced protein	9,5 e-21	defense response, pathogenesis, reduction of transcription
BvToc	355	tocopherol cyclase	1,6 e-22	response to oxidative stress, regulation of defense response
BvTum	507	translationally controlled tumour protein	8 e-41	cell cycle regulation, protection against stress
5 days-15 days post infection				
Bvaq	283	putative aquaporin	1,4 e-08	water transport
Bvchap	317	chaperone	7 e-06	response to stress
BvCytp	450	putative Cyt P450	1,7 e-50	detoxification by oxidoreduction
BvJac	781	jacalin like domain containing protein	3,4 e-08	defense response, apoptosis
BvJI	448	jasmonate-Induced protein	3,9 e-5	unknown function
BvPR10	793	pathogenesis related protein, family 10	3,6 e-35	putative RNAse activity
BvProt	567	proteasome alpha subunit	2,2 e-21	proteolysis, apoptosis
BvU1	750	-	4,1 e-25	cell cycle regulation
BvTrx	650	thioredoxin	2 e-03	response to ROS

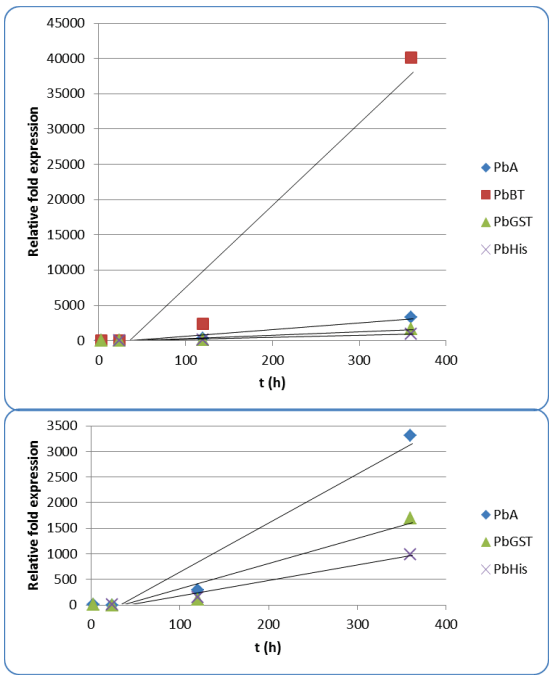

Fig. 3.1 : Over time relative expression of candidates housekeeping genes for *P. betae*. Values obtained 3 hours after the inoculation are taken as reference (value = 1) except for PbHis (24h).

Three of the four candidates as housekeeping genes (HKG) showed comparable increase rates: *P. betae* actin, glutathione-S-transferase and histone, with slopes of 9.57, 4.92 and 3.04, respectively. *P. betae* beta-tubulin, which showed a bigger increase rate of expression (slope = 116.42) was discarded from the housekeeping gene pool. The target gene 'histone' was discarded too, because no expression of this gene was detected 3 hours after the inoculation. The quantitative expression data of the five other *P. betae* tested genes were thus normalized using PbA and PbGST genes as housekeeping genes.

Clear variation in gene expression levels over the time were observed for two *P. betae* tested genes (fig. 3.2): the gene associated to the profilin (PbPro) and the gene coding for von Willebrand factor domain containing protein (PbvWf). The first one showed an increase of expression 24h post-inoculation while it was poorly expressed at the other times.

The same profile of gene expression was observed for PbvWf, with a peak after 24h and a net decrease after 5 and 15 days. The other tested genes showed less clear variation. A slight decrease of the expression of galactose-binding lectin was observed after 5 days, while at the same time, the gene coding for G protein seemed to be slightly overexpressed. The expression profile of the polysaccharide deacetylase showed a little peak after 24 hours.

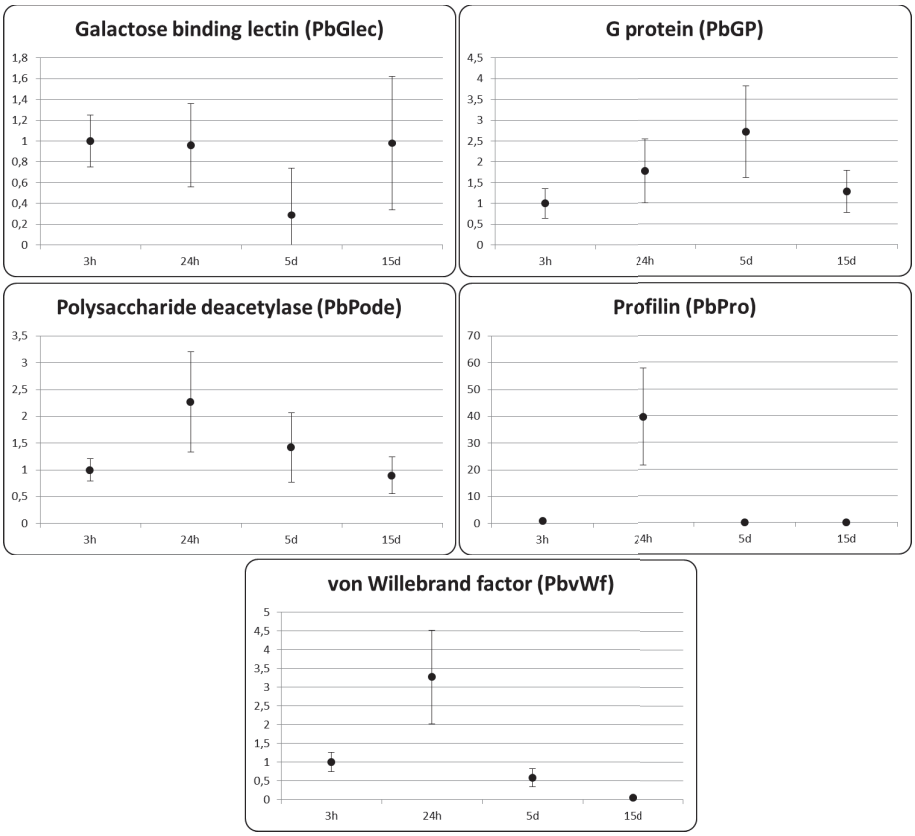

Fig. 3.2 : Relative expression of the *P. betae* genes. Values obtained 3 hours post-inoculation are taken as reference (value=1).

Sugar beet genes The dynamic of expression of the six tested sugar beet genes is presented on fig. 3.3 and 3.4. The majority of sugar beet

genes tested in this study were overexpressed in *P. betae* inoculated plants (Pb+) in comparison with the expression in *P. betae* uninoculated plants (Pb-), at least at one of the four tested times. However, the gene expressions showed different profiles. For 10 of the 11 tested sugar beet genes, gene expression levels in Pb+ and Pb- plants did not differ three hours after the inoculation time. A differential expression occurred for gene coding for "Salt-Induced protein" at this time, which exhibited a strong repression of transcription in Pb+. The expression of the genes coding for protease inhibitor (BvPin) and for cystein-rich protein (BvCys) increased over time when *P. betae* was present. The BvRem, BvRpolR, BvTum and BvPr10 genes were overexpressed in Pb+ after five days of infection and BvRip and BvProt one day and five days after infection. Finally, the expression profiles of two sugar beet genes strongly differed from the others, by exhibiting, most of time, a repression in Pb+ in comparison with Pb- plants. The expression of gene coding for jasmonate-induced protein (BvJI) was relatively similar with or without *P. betae*, except at 15 dpi where expression was repressed (4.5 fold less expression than control). Moreover, the gene coding for salt-induced protein (BvSI) showed a strong repression in Pb+ plants over time, except 24h dpi, where transcripts are more than seven fold more abundant than in Pb- plants.

3.1.4 Discussion

P. betae The first part of this study allowed the identification of 76 ESTs of *P. betae*. Thanks to these new sequences data, putative housekeeping genes were identified and tested in the second part of this work, as well as the expression of five *P. betae* genes. As shown in table 3.3, six *P. betae* ESTs encode putatively for proteins involved in signaling. Two of them, coding for distinct proteins 'PbGlec' and 'PbGlec2' and obtained during the infection phase or the endoparasitic phase respectively, are lectins. These glycoproteins are known to be involved in host-pathogen recognition (Vijayan & Chandra, 1999). Another *P. betae* EST involved in signaling encodes for a putative G-protein-coupled receptor (PbGP). Proteins from this family are often cell-surface receptors or cell-surface integral membrane proteins and are associated to pathogenicity in other pathosystems (Kulkarni *et al.*, 2005). The dis-

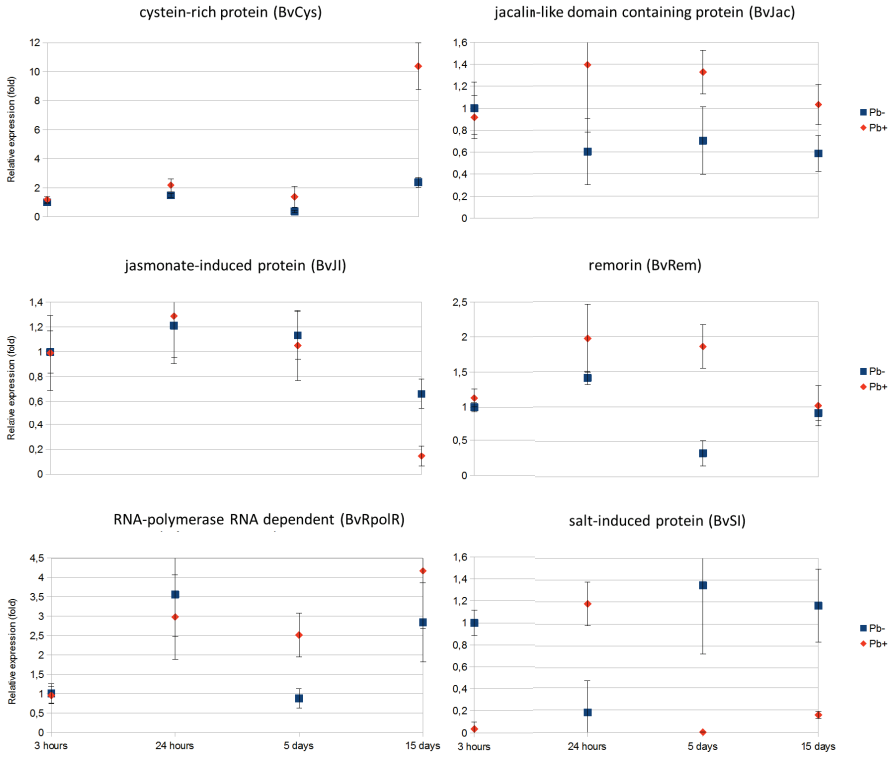

Fig. 3.3 : Relative expression profiles of six of the sugar beet tested genes in *P. betae* inoculated plants (Pb+) and in *P. betae* uninoculated plants (Pb-), normalized with BvGAPDH, BvBT and BvHis. Values obtained at 3 hours post inoculation in Pb- plants are taken as reference (value = 1)

ruption of gene coding for the α-subunit of the G-protein in *Fusarium oxysporum* led to a reduced pathogenicity (Jain *et al.*, 2002). The SH3 domain predicted to be contained in 'PbSH3' was associated with the signaling related to the cytoskeleton and involved in the yeast cellular rearrangements (Mirey *et al.*, 2005). This kind of activity could be useful for *P. betae* during the sporangial and sporogenic phases occuring 15 days after infection. Two other *P. betae*-predicted proteins contained domains such as SPRY or cystein-rich domains, 'Pbef1' and 'Pbef2'. Rehman *et al.* (2009) showed that secreted SPRY containing domain

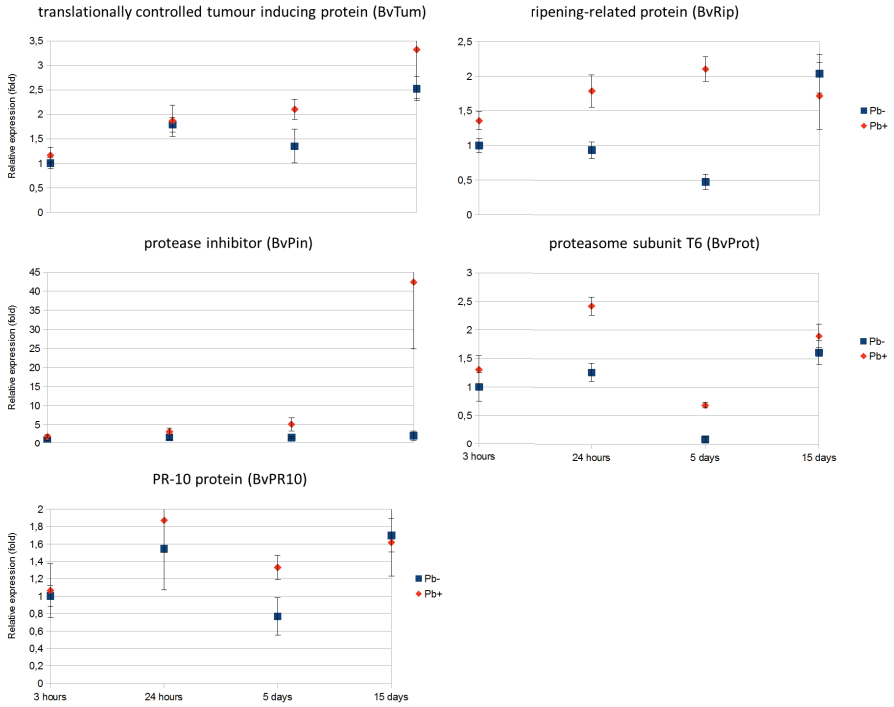

Fig. 3.4 : Relative expression profiles of five of the sugar beet tested genes in *P. betae* inoculated plants (Pb+) and in *P. betae* uninoculated plants (Pb-), normalized with BvGAPDH, BvBT and BvHis. Values obtained at 3 hours post inoculation in Pb- plants are taken as reference (value = 1)

proteins could be important effectors in the *Globodera rostochiensis*-tomato pathosystem, modulating the host defense responses. Most fungal avirulence genes (Avr genes) contain cystein-rich domains containing proteins (Stergiopoulos & De Wit, 2009). The corresponding coding-genes are supposed to facilitate the virulence of a parasite by suppressing the plant immunity, induced by pathogen associated molecular patterns (PAMPs) or involving the expression of resistance proteins in resistant plants (De Wit *et al.*, 2009). 'Pbef1' and 'Pbef2' could be thus involved in the dialogue between protist and plant and then included in the zigzag model of plant immune reactions (Jones & Dangl, 2006). Three putative polysaccharide deacetylases (PbPode, PbPode2, PbPode3) encoded

by three ESTs obtained during both periods of analysis were identified. Bulman *et al.* (2006) postulated that these kinds of proteins could help the cell-to-cell movement of *Plasmodiophora brassicae* by degrading the parietal components of the host cell, such as xylan. This could be the case for *Polymyxa betae* too because its biology is very close to the one of *Plasmodiophora brassicae*. Parts of sequences encoding for *Polymyxa betae* actin 'PbA' and β-tubulin 'PbBT' were identified in this study. These two partial gene sequences should be completed and used in a further phylogenetic analysis. It is worth noting that the PbvwF protein contains the von Willebrand factor domain. This domain, whose function is unknown in plasmodiophorids but involved in other protist infection process (Whittaker & Hynes, 2002), also appears in an EST encoded by *Plasmodiophora brassicae* (Bulman *et al.*, 2006). Many parallels can be made between the ESTs expressed by *Polymyxa betae* and *Plasmodiophora brassicae* during the infection in their respective host. Similar ESTs were sequenced in both plasmodiophorids, coding for proteins such as heat shocks proteins, histone 4 or β-tubulin. Concerning the putative response of pathogenicity, ESTs coding for glutathione-S-transferase and peptidase were sequenced in both protists. A sequence corresponding to a G protein was found in the both investigations. In the same way, transcripts for protein kinase and polysaccharide deacetylase were detected in both plasmodiophorids. These results revealed the great similarity between the *P. betae* - sugar beet interactions and the interactions within the clubroot model. This model offers new insights in the understanding of interactions *P. betae*- sugar beet, through the transposition of clubroot model to *P. betae* - sugar beet model. Five *P. betae* genes suspected to be involved in the infection and multiplication processes were chosen in order to establish their expression profile along the *P. betae* life cycle. Two of them provided particularly interesting profiles. The first is the gene coding for the profilin that was clearly overexpressed 24 hours after infection. The second is the gene PbvWf, exhibiting a significant increase of expression after 24h. Profilin is a cytoskeleton-binding protein. The overexpression of this gene observed after 24 h could be explained by the progression of *P. betae* within the cell. Another explanation could be that profilin is an essential determinant of pathogenicity, as revealed for other protists, human parasites, such as *Toxoplasma gondi*. It has been proven that this cell component

is essential for the invasion of the host cell (Plattner *et al.*, 2008). This event is occurring 24h after *P. betae* inoculation. Regarding the von Willebrand factor domain containing protein, such domain is contained in a protein produced during the infection phase of *Plasmodium falciparum*, the malaria causal protist agent (Whittaker & Hynes, 2002). The protein containing this domain in *P. betae* could thus play the same role since the overexpression occurred also during the infection phase. The expression of the gene coding for the lectin (PbGlec) decreases at five days. This lectin could act as receptor during first interactions between zoospores and plant. At five days, no more living zoospores are present, unlike the other tested times. This could explain the decrease of expression of this gene at this time. The polysaccharide deacetylase expression is relatively constant with a higher expression at 24 h. This profile could be explained by the putative action of this gene in cell-to-cell movement and cell penetration hypothesized by Bulman *et al.* (2006) for *Plasmodiophora brassicae*. The G protein, transmembrane receptor, is expressed by *P. betae* during the entire cycle but more intensively at 5 days. This protein could be produced in higher levels during membranes formation.

Sugar beet The table 3.4 presents the ESTs identified as sugar beet sequences. These ESTs correspond to proteins putatively interacting with *P. betae*. Most of them are proteins of defense and proteins of responses to stress. Albeit *P. betae* is asymptomatic, the infection by this parasite can stress the plant, which responds by the production of aggressive proteins and protection proteins ensuring its integrity. Pathogenesis-related like proteins were expressed, such as phenylalanine ammonia lyase (PAL) and lipid transfer protein as well as PR proteins (PR-10). These proteins are known to be defense proteins related to the systemic acquired resistance (SAR) (Van Loon *et al.*, 1994). Non SAR-dependent defense seems also to be involved interactions with *P. betae*. A RNA polymerase RNA-dependent and a subunit of a proteasome, predicted to be encoded by BvRpolR and BvProt, could respectively target *P. betae* by gene silencing and proteolysis. A putative remorin (BvRem) could be produced in order to alter cell-to-cell movement of *P. betae*, by acting on plasmodesmatas, as in the case of potato virus X (Raffaele *et al.*, 2009). Two other ESTs coding for antimicrobial compounds were identified: a protease inhibitor, and a cystein-rich protein,

putatively coding for gamma-thionin. A jacalin-like domain containing protein was encoded by ESTs sequenced at both times of analysis (BvJac). These proteins are known to be involved in plant defense and apoptosis (De Hoff *et al.*, 2009; Xiang *et al.*, 2011). In the case of an infection by the biotrophic *P. betae*, apoptosis is an interesting way to counter the protist progression. Moreover, cells self-destruction induces SAR, which could link this protein with the others previously cited, being the inducing event. Nevertheless, *P. betae* seems to by-pass this possible HR response. Cytochrome P450, putatively encoded by BvCytp, could act as detoxifying actor (Robineau *et al.*, 1998); chaperone and heat-shock protein (BvChap, BvHSP) are also proteins protecting the cell content when a stress occurs. Moreover, they were evidenced to be a part of the plant immunity system (Noël *et al.*, 2007). ESTs encoding for proteins regulating oxidative stress were identified: a putative cell redox homeostasis protein (BvHom), a ribosome associated membrane protein (BvRamp) and a tocopherol cyclase (BvToc), which has been evidenced to get an activity in plant defense regulation (Sattler *et al.*, 2006). These genes could be induced by the protist or to be the result of a plant oxidative response to a pathogen such as reactive oxygen species (Mittler *et al.*, 2004). Another stress of the plant during interaction could be a water stress. *P. betae* occupies cells of the absorbing roots which could reduce their ability of absorption. The presence of ESTs encoding for two water transporters could correspond to a compensation mechanism. Endly, unlike plasmodiophorids such as *Spongospora subterranea* on potato or *Plasmodiophora brassicae* on crucifers, *P. betae* is asymptomatic on sugar beet. This character could be due to the expression of genes in sugar beet in response to the infection by the protist. We hypothesize that the two ESTs putatively encoding for cell cycle regulation proteins, the translationally controlled tumour protein (BvTum) and the protein encoded by BvU1, also associated to the protection against stress, could be involved in this absence of symptoms.

The majority of sugar beet genes are overexpressed when *P. betae* is present at least at one of the four tested times. This result is logical given that the identification of the ESTs was done by SSH. The jasmonate-induced protein is not clearly overexpressed when the protist infects the plant, and this gene is even severely repressed (4.5 fold less

expressed than healthy controls) after fifteen days of infection. This profile could be due to the antagonism between the SAR and the induced systemic resistance, jasmonate-dependent. Jasmonic acid (JA) way is reduced when the salicylic acid way (SAR) is activated. The decrease in JA could logically reduce the jasmonate-induced protein. After three hours of infection, the first zoospores penetrated sugar beet root cells. At this time only two genes showed clear expression modifications: the putatives ripening-related (one of the PR10 proteins) and salt induced proteins. Transcripts of BvRip were about 1.5 fold more abundant in infected plants three hours after *P. betae* inoculation. Abundance of BvRip transcripts increased until the fifth day where Pb+ plants showed about 4.5 fold more expression of this gene. After fifteen days, the expressions in infected and healthy plants were similar. BvSI transcripts are reduced strongly even 3 hours after inoculation. This sugar beet gene was upregulated after 24 hours (6,6 fold more in Pb+ plants), then drastically repressed after five (770 fold less) and fifteen (7.4 fold less) days. The expression of this protein at certain stages of the life cycle of the protist could be harmful, and the expression could be stopped temporary by *P. betae*. The GO mapping showed that this protein had putatively a negative regulation of transcription activity. The repression of this protein by the plant could be done in order to upregulate the general level of transcription, needed to counter the protist infection. Between the first and the fifth days of infection, the protist grows into the cell and forms an amiboïd stage called plasmodium. Interestingly, at these two times, the majority of the plant defenses are upregulated. Before and during membrane formation, proteins such as proteasome could get access to protist protein in order to lyse these components. The transcripts coding for this protein are overexpressed after 24h (2 fold) and five days (7.8 fold). The RNA polymerase was upregulated three times after five days. siRNAs could interfere with the protist metabolism. This overexpression could be the sign of a gene silencing directed against the protist, a strategy of plant reported against viruses but not described against other pathogens (Vance & Vaucheret, 2001). After five days, the protist can expand in the plant by a cell-to-cell movement. The hypothese of remorin acting against this movement is consolidated by a three fold overexpression of remorin gene five days after *P. betae* inoculation. Curiously the second PR-10 protein (BvPR10)

gave a profile different from the ripening-related protein. Only a slight overexpression was denoted after five days (about 1.7 fold). The most probable explanation is that this protein is less active against *P. betae*. However, BvJac exhibited a profile with an overexpression between one and five days with about a 2 fold more transcripts in Pb+ plants. On the contrary of PR-10 protein, and remorin, jacalin-like domain containing protein seems to be directed against constant biological processes: its overexpression is not correlated with one life stage of *P. betae*. After fifteen days of infection, the majority of genes are expressed in the same range between Pb+ and Pb- plants. These results suggested that *P. betae* was hidden from the sugar beet defenses. However, two sugar beet genes are drastically overexpressed at this time. They encode for a cystein-rich protein (about 3.7 fold more after 5 days and 4.4 after 15 days) and a protease inhibitor (2.2 fold more after 5 days and 19.6 fold more after 15 days) when *P. betae* is present. As previously said cystein-rich protein is probably a protein from the gamma-thionin family. These proteins functions principally as membrane disruptors (Stotz *et al.*, 2009). Before five days the production of this protein could be useless because *P. betae* is in its growth phase, membrane is probably under construction. The same profile exhibited by the protease could correspond to a response to a protease secreted by *P. betae*.
In the pool 5-15 days of sequences obtained after SSH analysis, a putative peptidase was produced by *P. betae*. This protein could be the target of the protease inhibitor. Except these two genes, BvCys and BvPin, other gene expressions were not up or down regulated regarding to the controls. At this time, *P. betae* could "hidden" itself, in an immunitary view. Plant defenses seem to be less active, the battle seems to be over. After fifteen days of *P. betae* infection, survival spores and zoosporangia are formed. The survival spores possess logically a decreased activity and the multiple cell wall layers of the sporosori can protect the protist against plant defenses. An interesting hypothesis could be that aggressive proteins such as BvCys and BvPin are involved in the formation of the first sporosori. Sporosori usually forms when a lot of cell roots are infected by the protist. After fifteen days of infection, no such infection is observed, but some sporosori are formed, which could putatively be the result of a protection of *P. betae* against these two proteins. Observation of *P. betae* behaviour in sugar beet plants overexpressing these

two genes could be useful in order to confirm the hypothesis of their involvement in sporosori formation. Nevertheless, this decrease of plant defense activity occurs in the same time of a very active stage of *P. betae* life cycle, the zoosporangia. The most active times of interaction between the plant and the protist seem thus to be situated between one day and five days after inoculation. This time corresponds likely to the period of interaction where the major stresses for both actors occur. This could explain the overexpression of protective protein BvTum.
This paper offers new insights in the understanding of sugar beet-*P. betae* interactions. First, 76 ESTs of *P. betae* were sequenced. The study of the dynamic of genes expression during *P. betae* life cycle revealed that two genes were correlated with the infection phase, 24 hours after inoculation (PbPro and PbvWf). One of these genes is a determinant of pathogenicity in another protist species, *Toxoplasma gondii*. At this time, the cytoplasm of the two organisms are in contact. A knock-down approach, using RNA silencing or a replicon of BNYVV transmitted by the protist itself (Schmidlin *et al.*, 2005) could be useful to check the importance of these genes in *P. betae* establishment. The analysis of profile of expression of eleven sugar beet genes revealed an overall overexpression of defense and protection genes that began one day after infection and was at the maximum at five days. This period of time seemed to be a very active defense phase, with the majority of plant defenses genes activated. After fifteen days, when zoosporangia and sporosori are formed, only two defense genes, probably directed against one of these two life stages, were overexpressed. At this time, interactions between the two organisms seemed quieter, as if *P. betae* was "immunitary hidden". Finally, various SAR-dependent proteins were evidenced. Even three hours after inoculation, the systemic resistance way was induced by the protist. The induction of the SAR by *P. betae* could even be benefic for the sugar beet by acting against other pathogens and should be evaluated in further studies. The entire sequences of *P. betae* genes should also be completed and their putative role assessed using functional gene analysis methods.

CHAPTER 4

INTERACTION BETWEEN *POLYMYXA BETAE* AND PLANT SYSTEMIC DEFENSE WAYS

Opening comments

In the chapter 3, ESTs sequencing revealed evidence of mechanisms of specific resistance and production of pathogenesis related proteins, typical of systemic resistance ways. It seemed thus interesting to test the interaction between *P. betae* and the two systemic resistance ways of the plant (SAR/ISR). In a first experiment, we tested if the elicitation of induced systemic resistance of the plant was effective or not against *P. betae* infection. By this way, we tried to know if sugar beet had potentially the means to counter *P. betae*. In a second experiment, we evaluated if the systemic resistance conferred by *P. betae* allowed the plant to be protected against other pathogens. This last experiment evaluated if an "immune" benefit can be given by *P. betae*.

4.1 Systemic resistance induced by *Bacillus* lipopeptides in *Beta vulgaris* reduces infection by the rhizomania disease vector *Polymyxa betae*

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4.1.1 Introduction

Polymyxa betae Keskin (Keskin, 1964) belongs to the plasmodiophorids, which is now included in the *Phytomyxea* in the phylum *Cercozoa* (Adl *et al.*, 2005; Bass *et al.*, 2009). It is an obligate biotrophic parasite of the roots of sugar beet, acting as a vector of the rhizomania disease of sugar beet caused by *Beet necrotic yellow vein virus* (BNYVV). Until now, the control of this major disease has been managed through varietal resistance to BNYVV. The control of *P. betae* in the field remains limited to cultural practices (Goffart & Maraite, 1991) because effective chemical treatments, such as methyl bromide, have been forbidden (UNEP (United Nations Environment Programme), 1987). Barr *et al.* (1995) and Asher *et al.* (2009) have shown the potential of breeding sugar beet for protist resistance from the resistance genes of wild *Beta* species, but this approach is still not used by breeders because of lower productivity and the unknown long-term impact of these varieties on the disease. In this context, biological control of the protist vector of rhizomania could be an interesting alternative to breeding for reducing disease pressure. In many instances, biological control of microbial diseases is obtained after the inoculation of plant beneficial organisms that will directly hamper development of the targeted pathogens. Another interesting biocontrol mechanism relies on the stimulation of the natural defenses of the host

plant by these beneficial microbes or by their products. Plants might develop various strategies for combating aggressors (Van Loon *et al.*, 1998). One of these strategies is a defense reaction in the tissues surrounding the initial infection site, a phenomenon known as 'localized acquired resistance'. Such an elevated resistance level, however, can spread throughout the plant via the emission of molecular signals that will reach distal tissues, rendering the whole plant less susceptible to subsequent pathogen attack. This phenomenon, widely reviewed in recent years, is known as 'systemic acquired resistance' (SAR). It is commonly triggered by the elicitors of avirulent pathogens, such as microbial-associated molecular patterns (MAMPs) (Abramovitch *et al.*, 2006), but it can also be induced by biological (non-microbial) and chemical compounds. In the biocontrol context, another interesting form of systemic resistance in plants is the one which is referred here as ISR (induced systemic resistance) and induced by non pathogenic, but plant growth-promoting microorganisms, including fungi (PGPFs) and rhizobacteria (PGPRs). Among the PGPRs, species in the *Pseudomonas* and *Bacillus* genera are the most well known (Raaijmakers *et al.*, 2010). Phenotypically, ISR is quite similar to SAR, making the plant resistant to subsequent attacks of pathogenic organisms such as viruses, bacteria and fungi (Bakker *et al.*, 2007). The signalling of these systemic resistances is controlled by salicylic acid (SA), jasmonic acid (JA) and ethylene (ET), with SA involved in the case of SAR, and JA and ET associated mainly with signalling of ISR. These two apparently independent signalling routes contain crosstalks and converge through the same transcriptional regulator, NPR-1 (Shah, 2009; Katagiri & Tsuda, 2010). The systemic resistances do not confer total resistance to a pathogen, but they provide long-lasting increased resistance in a great number of plants against a broad range of pathogens. Some chemicals, such as SA or analogs (BTH, benzodiathiazole and derivatives, 2,6-dichloro-nicotinic acid), are known to induce SAR and have been successfully used in the field to control diseases (Vallad & Goodman, 2004). With regard to ISR, field or greenhouse trials with inducing organisms have shown its potential for controlling several diseases (Bent, 2005; Kloepper *et al.*, 2004). For sugar beet, systemic resistance induced by non-pathogenic species was tested successfully in two pathosystems: *Pseudomonas fluorescens* enabled the control of *Heterodera schachtii* (Bargabus *et al.*, 2002), whereas *Bacillus mycoides* and

B. pumilus efficiently controlled *Cercospora beticola* (Bargabus *et al.*, 2004). Based on many promising results, research was conducted on developing microbial formulations that could be used in agriculture. Two classes of bacterial biosurfactants were found to be elicitors of ISR: rhamnolipids and cyclic lipopeptides (cLPs). cLPs produced by *Pseudomonas* and *Bacillus* were shown to elicit ISR. Massetolide A from *Pseudomonas fluorescens* elicited ISR and enabled *Phytophthora infestans* on tomato to be controlled (Tran *et al.*, 2007). Pure fengycins and surfactins triggered a significant protective effect, similar to that induced by the producing *Bacillus* strains. In addition, over-expression of surfactin and fengycin genes in poor cLPs-producing strains was associated with a higher level of resistance (Ongena *et al.*, 2007). The ISR activity of surfactin was associated, in treated plants, with the accumulation of antifungal compounds (phytoalexins) (Adam, 2008) and with the stimulation of the lipoxygenase pathway leading to the synthesis of fungitoxic oxylipins (Ongena *et al.*, 2007). The mechanism of ISR by lipopeptides is not yet clear, but a recent study strongly suggests that the plant cell recognition of surfactin is mediated through interaction with lipids at the plasma membrane level, rather than through specific protein receptors (Henry *et al.*, 2011). This lipid bilayer perturbation does not affect cell viability, but is enough to trigger a cascade of molecular events leading to an increased defense response (Jourdan *et al.*, 2009). In order to prevent rhizomania in sugar beet more efficiently, it is necessary to combine classical strategies, such as breeding varieties that can resist the virus with controlling the vector of the disease. This study sought to evaluate the potential of controlling *P. betae* by inducing systemic resistance in sugar beet using *Bacillus* cLPs.

4.1.2 Material and Methods

Plant growth and elicitation

The potential of using *Bacillus* cLPs to induce systemic resistance in sugar beet against *P. betae* infection was tested in a bioassay conducted under controlled conditions. Pre-germinated seeds of sugar beet (var. Cadyx) were transferred in individual glass tubes containing sterilized quartz, and were incubated in a growth chamber with a 14 h day/10 h night photoperiod, with temperatures of 25°C and 20°C, respectively.

The plants were watered every 2 days with Hoagland solution, pH 7.2. Semi-purified cLPs (80% purity established by reverse phase HPLC coupled with a single quadrupole mass spectrometer) produced by *B. amylolequifaciens* isolate S499 were used for the plant treatment. This extract contained a mixture of compounds belonging to the three cLPs families - surfactin, iturin and fengycin - produced by isolate S499 in the relative proportions of 55/22/23 v/v/v, respectively. The extract was produced after growth under laboratory conditions in a medium that had been established for the enhanced production of such compounds. Secreted cLPs were submitted to acid precipitation and solid phase extraction on a C18 cartridge in order to recover an 80% pure solution as determined by reverse phase HPLC coupled with electrospray ionization mass spectrometry, following a procedure described by Nihorimbere *et al.* (2011). The solution of cLPs for plant treatment was prepared by diluting the methanolic lipopeptide extract with sterile milliQ water. Two treatments were applied three weeks and one month after plant germination: each plant was watered with 5 ml of cLPs solution (60 mg/l) and 5 ml of Hoagland solution, pH 7.2. The final concentration of LPs in the solution surrounding the roots was 30 mg/l. The control plants were treated with 5 ml of sterile milliQ water and 5 ml Hoagland water, pH 7.2. Twenty-four plants were treated with each concentration (0 mg/l [controls] and 30 mg/l).

Zoospores inoculation

Fourteen days after the first treatment with cLPs or water, 20 plants per treatment were inoculated with *P. betae* zoospores. In order to obtain the required mobile stage, the aviruliferous monosporosorus *P. betae* isolate A_{26-41} collected from a rhizomania-free field at Opprebais in Belgium in 1987 was used (Legrève *et al.*, 1998). The multiplication of this isolate was achieved by growing sugar beet (*Beta vulgaris* var. Cadyx) plants on a quartz-sporosori mixture using an automatic immersion system in an environmental cabinet at 20-25°C, as described by Legrève *et al.* (1998). Large quantities of zoospores were obtained from the roots of young plants, as described by Desoignies *et al.* (2011). Two suspensions of 100 and 1000 zoospores/ml were prepared for inoculating plants treated with cLPs or water. Ten plants per treatment were inoculated

with 500 zoospores each and another 10 with 5,000 zoospores each. After inoculation, the quartz substrate was saturated with Hoagland solution, pH 7.2.

Plant harvest and *Polymyxa betae* quantitation

The elicited but uninoculated plants were harvested on the day the other plants were inoculated. The inoculated plants (cLPs- and water-treated) were collected seven days after inoculation with *P. betae* zoospores. For the harvest, each root system was rinsed in demineralized water, and 200 mg of fresh tissues were collected from each root system. Nucleic acids were extracted in one ml of polysomes buffer (Jupin *et al.*, 1990) in 2 ml microtubes with 1/4" ceramic spheres using the FastPrep© instrument (Qbiogene, CA, USA). This step was followed by phenol extraction and ethanol precipitation. We wanted to test whether the cLPs treatment could have an impact on the infection of sugar beet by *P. betae*. Using quantitative PCR, the infection was evaluated seven days after inoculation with two concentrations of zoospores (500 and 5,000 per plant). A qPCR was conducted on the root extracts of *P. betae*-inoculated plants with or without previous cLP treatment. SYBR-Green chemistry was used in this study. Two duplicates of the qPCR were made for each sample, standard, control or blank. The reaction components for each sample were 20 μl iQ SYBR Green Supermix, 0.8 μl of each primer (20 pmol) (primers used are shown in Table 4.1), 14.4 μl of DEPC water and 4 μl of the 20-fold diluted DNA extract. Amplification reaction was performed using the iCycler iQ Real Time detection system of Biorad, as follows: first, the denaturation step at 95°C for 3 min, then 40 cycles of 30s at 95°C, 30s at 62°C and 30 s at 72°C, and finally a melting curve was established. The DNA standards for *Beta vulgaris* glutamine synthetase were obtained from serial dilutions of a phenol/chloroform extraction of 500 mg of fresh foliar tissues. For *P. betae* standards, PCR products were cloned in PGEM-T vector (Promega). Serial dilutions of these two standards were used to construct the standard curves for the different quantification experiments. The detection threshold was fixed at a fluorescence of 150 for *P. betae* quantitation and at a fluorescence of 250 for sugar beet glutamine synthase. The quantification cycles (Cq) were used to compare the different samples. A mean was calculated

for the two replicates of each reaction. The relative Cq was calculated, corresponding to the quantity of *P. betae* per sugar beet cell unit: for the same extract, the Cq corresponding to the two replicates of qPCR targeting the *P. betae* sequence was divided by the Cq corresponding to the two replicates of qPCR for sugar beet glutamine synthetase. For each plant tested, the value of relative Cq was then obtained. For each concentration of zoospores, an ANOVA-1 was applied, with the concentration in cLPs as the explicative variable. Various statistical tests of mean comparison (Tukey, Dunett, Scheffe and SNK) were applied between the elicited and non-elicited plants. All these tests were done using SAS enterprise guide 2.0 (SAS, Cary, USA). A replication of the whole experiment was performed.

Table 4.1: Primers used. The primers were designed with ePrimer (NCBI)

Organism	Targeted region	Use	Primer name	Sequence	References
Polymyxa betae	Internal transcribed spacer 1	qPCR	Pb1	5'-GGAATTTGAACAAGTGACTTGG-3'	Legrève *et al.* (2003)
			Psp2rev	5'- AGGGCTCTCGAAAGCGCAA-3'	
Beta vulgaris	Glutamine synthetase	qPCR	GSBvFor	5'-AGGGTGATTGGAATGGTGCT-3'	This study
			GSBvRev	5'-ACTTCTCGATGGCAGCCTTT-3'	
Beta vulgaris	*NPR-1* Gene	RT-PCR	NPR1BvF	5'-TCATGAAGCTTGTCGTCCTG-3'	This study
			NPR1BvR	5'-ATACACCTTGCCAGCAATCC-3'	
Beta vulgaris	Chitinase Class III	RT-PCR	Chit3BvF	5'-GCTGAACTTAGCTGGGCACT-3'	This study
			Chit3BvR	5'-CTGGACTGACCCCCAAGATA-3'	

Assessment of NPR-1 and PR-8 expression

RT-PCR was performed on cLPs- and water-treated control plants when the other plants were then inoculated. Nucleic acids from roots and leaves tissues of control plants (cLPs- and water-treated but uninoculated) were extracted as described previously. DNase treatment was applied to prevent false positives in RT-PCR. Then, seven μl of 20-fold diluted samples were digested with 2.5 μl RQ1 DNAse (Promega, Madison, USA), following the manufacturer's protocol. cDNA synthesis was performed in two steps: first, a mix of 1 μl reverse primer (primers used are shown in Table 4.1), 8.5 μl of DEPC water and 1 μl RNA was incubated for 10 min at 65°C. Second, a reaction mixture of 4 μl of M-MLV RT buffer, 0.25 μl of M-MLV reverse transcriptase (200 U/ μl) (Promega), 2 μl of dNTP (20 nmol) and 3.5 μl of DEPC water was added to the first reaction mix and incubated at 42°C for 60 min. The cycling times and temperature for the RT-PCR detection were 94°C for 2 min (1 cycle) 94°C for 30 s, 60°C for 30 s, 72°C for 30 s (35 cycles) and 72°C for 1 min (1 cycle). The reaction components for each sample were 13.25 μl DEPC water, 2.5 μl MgCl2 (25 mM), 5 μl of Green GoTaq Flexi buffer (Promega), 0.75 μl dNTP (20nmol), 0.5 μl of each primer (20 pmol), 0.125 μl of GoTaq Polymerase (Promega) and 2.5 μl cDNA. After ethidium bromide staining, DNA bands were visualized using Gel Doc 2000 (Biorad, Hercules, CA, USA).

Assessment of direct action of cLPs on *P. betae* zoospores

The concentration of a freshly prepared zoospores suspension was adjusted to 75,000 zoospores/ml just before the addition of cLPs at final concentrations of 0 (control), 30, 60 and 120 mg/l. Zoospore integrity was measured in five replicates of 1 ml of each suspension by quantifying ATP via a luminescent assay. After three hours of incubation, the viability of *P. betae* zoospores was assessed by ATP quantitation of each sample with the Cell-Titer Glo kit (Promega), following the manufacturer's protocol. The luciferase activity was measured with a Varioskan Flash Multimode Reader (Thermo Scientific, Waltham, USA).

4.1.3 Results and discussion

Our data showed that the treatment with cLPs significantly reduced the infection of plants by *P. betae* compared with the negative controls (water-treated plants): the mean of relative Cq increased from 1.131 to 1.326 (repetition 1) and from 1.444 to 1.536 (repetition 2) when 500 zoospores were inoculated, and from 1.018 to 1.169 (repetition 1) and from 1.352 to 1.522 (repetition 2) when 5,000 zoospores were inoculated. All comparisons of means using SNK, Tukey, Scheffe and Dunett tests showed a significant difference. A boxplot of the dispersion of the relative Cq for the first repetition is shown on figure 4.1. These results mean that infection was reduced by more than 93% (repetition 1) and 74% (repetition 2) when 500 zoospores were inoculated and by more than 88% (repetition 1) and 93% (repetition 2) when 5,000 zoospores were inoculated (table 4.2).

Table 4.2: Reduction of *P. betae* infection rates in cLPs-treated plants. The reduction rates are reported as the difference between *P. betae*-Relative Cq of treated plants and *P. betae*-Relative Cq of the controls multiplied by the mean of the *Beta vulgaris* Cq. It represents the standardized difference in Cqs. At each amplification cycle, DNA quantity is multiplied by two. The reduction factor can be obtained as $2^{\Delta Cycles}$. Finally, the reduction rate is obtained as follows: reduction rate = 1 -1/Reduction factor.

Zoospores inoculated (Replicate)	Differences in *P. betae* Relative Cq	Mean sugar beet Cq	ΔCycles	Reduction factor	Reduction rate
500 (1)	0.195	20.436	3.98	15.78	93.66
5,000 (1)	0.151	20.32	3.07	8.39	88.08
500 (2)	0.092	21.6	1.99	3.97	74.82
5,000(2)	0.169	21.61	3.65	15.67	93.61

cLPs are then active against *P. betae* infection, but their mode of action has not yet been shown. The most probable is the cLPs action of elicitation of plant systemic resistance. In order to evidence these hypothesis, the expression of genes coding for the NPR-1 transcription factor (non-expressor of pathogenesis related genes) in the roots and leaves and for a PR-8 protein (roots) was assessed by RT-PCR on control plants harvested the day of zoospores inoculation. NPR-1 was selected

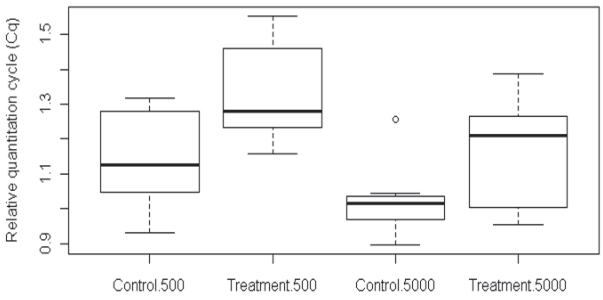

Fig. 4.1 : Boxplot of relative Cq (Cq *Polymyxa betae*/Cq *Beta vulgaris*) obtained by qPCR for the quantification of infection rate of sugar beet by *P. betae*. Control refers to unelicited plants; treatment refers to elicitation with lipopeptides (30 mg/l); 500 or 5,000 were the number of *P. betae* zoospores inoculated per plant. N=10

because it plays a key role in signalling both SAR and ISR pathways. PR-8 is a chitinase class III defense enzyme that could antagonize the growth of *P. betae* containing chitin. In addition, this gene is controlled by NPR-1, confirming the action of NPR-1 as a transcription activator. The expression of these genes was assessed at the time of the inoculation with *P. betae* in order to check whether or not systemic resistance was effective in cLPs- and water-treated plants.

cLPs-treated plants showed high expression of the tested genes compared with controls. The results showed that the two tested genes were over-expressed in roots after treatment with cLPs compared with non-elicited (water-treated) plants (Figure 4.2). NPR-1 was not expressed in untreated roots, whereas PR-8 was detected, but the RT-PCR showed that the presence of cLPs led to a much higher expression of PR-8. The strong accumulation of NPR-1 in the leaves of plants with cLP-treated roots clearly demonstrated the systemic nature of cLP-induced resistance in sugar beets. All these data strongly suggest that the penetration or further multiplication of *P. betae* in the root tissues of elicited plants was severely impaired. As the addition of cLPs and infection by *P. betae* were performed on the same plant organ, however, these biosurfactants might have directly inhibited zoospore performance. In order

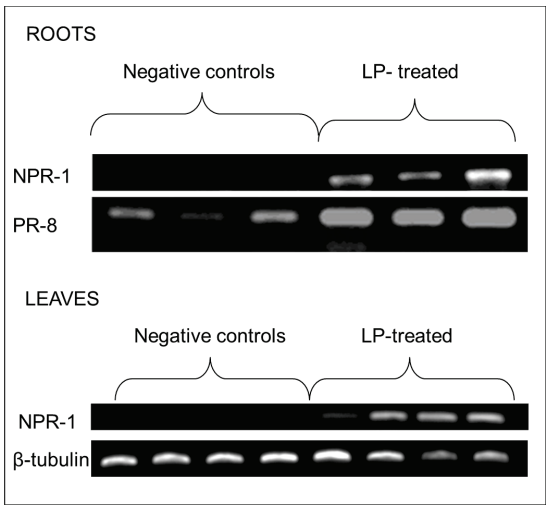

Fig. 4.2 : Expression of *NPR-1* and *PR-8* in roots and *NPR-1* and sugar beet β-tubulin in leaves of sugar beet plants, treated or not treated (negative control) with lipopeptides 7 and 14 days before. Three (roots) or four (leaves) plants were tested per treatment.

to test this hypothesis, the viability of the zoospores was measured after 3 h of contact with varying concentrations of cLPs (Table 4.3).

No significant difference (tests SNK, Dunett, Tukey and Scheffe) was evident among the luciferase activities from the zoospore suspensions prepared in the 3 cLPs concentrations and the control, suggesting that the zoosporicidal activity of cLPs is very low and cannot explain the protective effect observed in biocontrol assays on whole plants. Although occasionally suggested, there are few reports showing direct lytic activity of rhizobacterial cLPs on the zoospores of soil-inhabiting protists in general (Jousset *et al.*, 2006; Nielsen *et al.*, 1999). The results obtained in this study show that the elicitation of sugar beet with cLPs from *B. amylolequifaciens* prior to inoculation with *P. betae* reduced infection by this parasite. So far as we know, this antagonistic effect on plasmodiophorids has not previously been demonstrated. cLPs treatment confers partial resistance to *P. betae* infection, probably by inducing systemic resistance in sugar beet. Our results indicated that this involves the transcription activator NPR-1, which does not allow discrimination between the SAR-

Table 4.3: Quantification of zoospore viability in the presence of cLPs. The viability of zoospores was measured throughout the ATP quantitation process in a solution containing the same number of zoospores but increasing concentrations of lipopeptides.

Concentration in cLPs	N	Luciferase activity, relative measure	
		Mean	Standard deviation
0 mg/l	5	31,272.31	8,735.19
30 mg/l	5	40,317.13	8,243.71
60 mg/l	5	31,469.70	7,926.97
120 mg/l	5	32,416.49	6,708.03

and ISR-type responses stimulated by these compounds. Stimulation of the pathogenesis-related chitinase PR-8 indicates that a SAR-like reaction could be involved, but more work is needed to better understand the molecular basis of this protective effect. Indeed, there is no clear boundary between ISR and SAR, which are connected by cross-talk, and the expressed PR proteins could be a sign of induced SAR or, in contrast, of a primed defense that is often the result of ISR (Conrath *et al.*, 2002). This study also extends the known range of plant species in which *Bacillus* cLPs are active at stimulating a systemic resistance response already observed in tomato, bean and tobacco (Ongena & Jacques, 2008). This therefore also reinforces the notion that such biosurfactant compounds constitute a new class of MAMPs, recognized by plant root cells (Ongena *et al.*, 2007). Moreover, Henry and colleagues (2011) showed that surfactin is involved in the elicitation process, through a lipid-driven process at the plasma membrane level. We can conclude that systemic resistance, induced by *Bacillus* cLPs, drastically reduces the infection of sugar beet by *P. betae*. Barr *et al.* (1995) and McGrann *et al.* (2009*b*)) have shown that the partial resistance of sugar beet to *P. betae* is linked to reduced virus levels. These conclusions indicate that disease pressure could be reduced by decreasing the infection pressure of the BNYVV vector. cLPs appear to offer a new method for the biocontrol of rhizomania. Seed coating with such cLPs could enhance the priming in sugar beet seedlings, and by this way reduce *P. betae* and rhizomania pressure. Assays in fields or using *P. betae* infected soils should be conducted in order to test this hypothesis.

4.2 Assessment of eliciting abilities of *P. betae*

4.2.1 Introduction

During a master thesis (Schramme, 2010), we evidenced the elicitation of systemic resistance way by *Polymyxa betae*. Even if the inducing molecule (chitin, flagelle component) and mechanism remain to be discovered, plants infected by *P. betae* exhibited a higher expression of *PR-1a*, a classical marker of the SAR induction (Van Loon & Van Strien, 1999). All parasites, as well pathogens as mutualists, harm their host to foster their own needs. The cost of parasitism is not known in the interaction *P. betae* - sugar beet. But it can be balanced by benefits of other nature, not evident and visible, the "hidden benefits" (Leung & Poulin, 2008). The SAR elicited by *P. betae* could be a "hidden benefit" and help the plant to prevent infection by harmful pathogens. *P. betae* can not be a commercially exploited elicitor, given its ubiquitous nature and its ability to transmit BNYVV. Nevertheless, the investigation of the putative benefits is interesting in order to respond to the remaining question concerning the kind of parasitism of *P. betae*. In order to test the potential elicitor effect of *P. betae*, we analyzed the impact of a pre-infection of *B. vulgaris* by *P. betae* on a subsequent infection by *Cercospora beticola*. This pathogen was chosen for two reasons. First, infection by *C. beticola* is reduced by the two systemic resistance ways. Indeed, Bargabus *et al.* (2002) showed that Cercospora leaf spot (CLS) was reduced up to 79% when a systemic resistance was induced using acybenzolar-S-methyl or *Bacillus mycoides* (see tab. 4.4). Secondly, the

Table 4.4: Impact of induction of systemic resistances by acybenzolar-S-methyl (ASM), a salicylic acid analog, and *Bacillus mycoides*, a non-pathogenic rhizosphere bacteria, on the development of Cercospora leaf spot, fourteen and twenty one days post-infection (dpi). Adapted from Bargabus *et al.* (2002).

	% Disease severity		
Treatment	14 dpi	21 dpi	% Disease reduction (21 dpi)
Control	5.24	14.10	-
ASM	0.76	5.26	63.6
B. mycoides	1.03	2.94	79.5

foliar nature of *C. beticola* facilitates the visualization of the potential

P. betae-induced systemic resistance: *P. betae* is inoculated to the roots and the tested pathogen to the leaves. Moreover, disease quantitation is more easy and direct interactions such as antagonisms can be excluded.

4.2.2 Material and Methods

Sugar beets plants Sugar beets seeds var. Cadyx were pregerminated at 25°C in the dark on humid paper for two days. Seedlings were then transferred in PVC tubes adapted to automatic immersion systems (AIS), as described by Legrève *et al.* (1998). Sixty plants were grown. AIS were placed in controlled environmental rooms at 20-25°C, with a photoperiod of 14h. The automatic immersion system allowed alternate flooding-drainage periods (12 hours flooding/12 hours drainage) regulated by an electric timer.

***Polymyxa betae* inoculum** The aviruliferous monosporosorus isolate A_{26-41} from a soil collected in a non-rhizomania-infested field at Opprebais in Belgium in 1987 was used in this study (Legrève *et al.*, 1998). The multiplication of this isolate was achieved as described earlier. A huge number of zoospores was produced as described by Desoignies *et al.* (2011).

***Cercospora beticola* inoculum** The fungal inoculum source consisted in a mixture of three isolates collected on sugar beets from Belgium and France. Two successive cultures of this mixture were done, on V8 medium, at pH 7.5 and pH 5.0, respectively, and conidies were harvested, as described by De Coninck *et al.* (2011). Conidies were conserved at $-80°C$.

Bioassay Sugar beets were grown for five weeks in AIS. Eighteen plants were then inoculated with 5,000 *P. betae* zoospores each (Pb+ plants). Another set of eighteen plants were not inoculated with *P. betae* (Pb- plants). Two weeks later, Pb+ and Pb- plants were challenged with *C. beticola*, using the protocol described by De Coninck *et al.* (2011). The concentration of conidies solution was adjusted to 3.10^4 conidies/ml with sterile water and plants were spray-inoculated until runoff with this suspension. Immediately after inoculation, plants were kept for sixteen

days at 25/20°C, 14 h photoperiod and in a high humidity with a dampener. After sixteen days, plants were harvested. The roots were stained by boiling for 2 min in lactophenol blue to assess the presence of *P. betae* by microscopy.

Cercospora leaf spot disease quantification and statistical analysis The progress of the Cercospora leaf spot on each plant was quantified nine, twelve, fourteen and sixteen days post inoculation. For each plant and time, the most diseased leaf was chosen and scanned using a scanner CANON Canoscan. The quantification of the disease lesion was done using the image analysis software "Assess", as described in the user manual (Lamari, 2002) (ASSESS; L. Lamari, American Phytopathological Society,St. Paul, MN, USA). ANOVA-2 and means comparisons were done using "R" (R Development Core Team, 2012).

4.2.3 Results

Cercospora leaf spot incidence Nine days after the inoculation of *C. beticola*, CLS symptoms were observed on fifteen Pb- plants and only three Pb+ plants (fig. 4.3). The number of plants showing Cercospora leaf spots (CLS) symptoms was thus strongly different depending on whether the plants were previously inoculated by *P. betae* or not. Afterwards, the difference in number of diseased plants remained strong: at twelve dpi, all Pb- plants were diseased whereas only the half of Pb+ plants showed symptoms. At 16 dpi, three Pb+ plants remained asymptomatic.

Global aspects of the plants The first symptoms were then observed 9 dpi. Twelve dpi, severe symptoms were visible on Pb- plants whereas only small spots were observed on Pb+ plants (fig.4.4). Fourteen dpi, Pb- plants showed very damaged leaves, with large lesions almost covering the entire surface of leaves. It is only at 14 dpi that spots appeared on several leaves of Pb+ plants.

CLS severity assessment The progress of the CLS severity (diseased area) during sixteen days after infection is shown in figure 4.5 for Pb+ and Pb- plants. At each time of observation, the mean percentage of

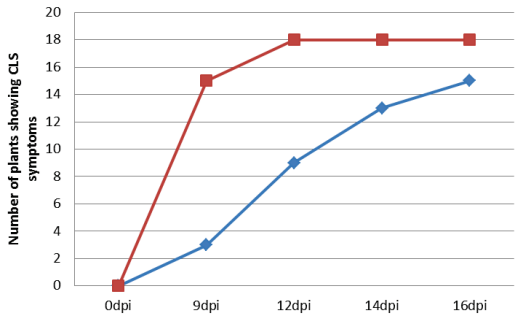

Fig. 4.3 : Progress of CLS incidence on plants previously inoculated (blue) with *P. betae* (Pb+) and on plants non-inoculated (red) with *P. betae* (Pb-)

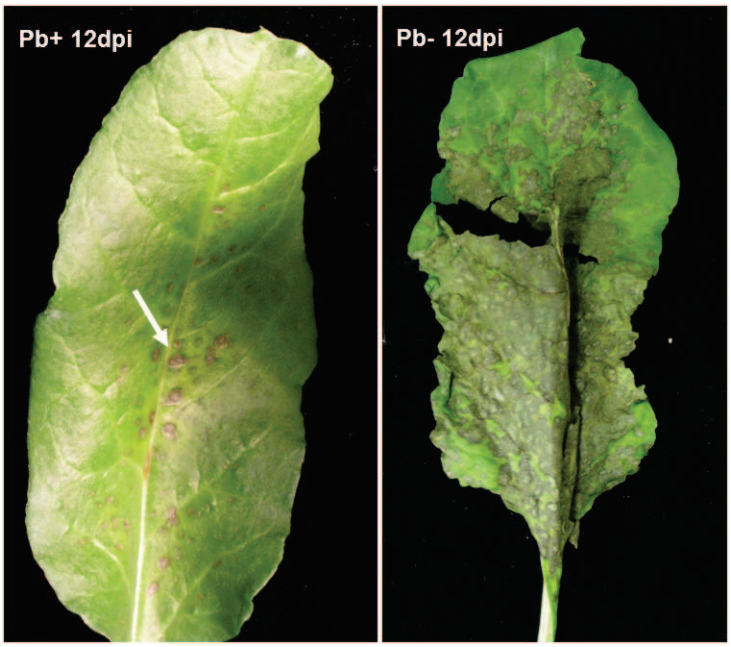

Fig. 4.4 : CLS symptoms observed on sugar beet leaves, twelve days after *Cercospora beticola* inoculation, on plants previously inoculated (Pb+) or not (Pb-) with *P. betae*

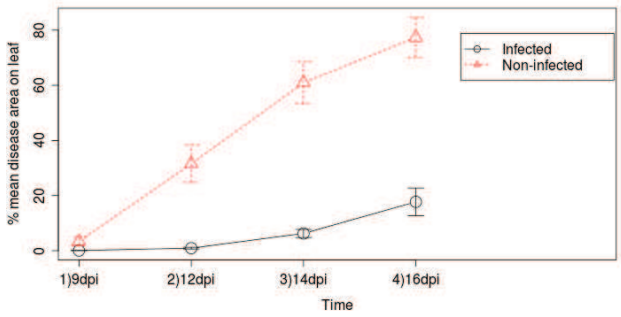

Fig. 4.5 : Mean of disease area of Cercospora leaf spot on the most damaged leaves when plants are previously infected by *P. betae* (circles) or not (triangles).

lesion area was significantly different between Pb+ and Pb- plants. At 16 dpi, diseased leaf area mean of Pb+ plants is still under the values of Pb- plants at nine dpi. The disease reduction conferred by *P. betae* was calculated at each time (tab. 4.5) At the last observation (at 16 dpi), the mean diseased area of Pb+ plants are about 4.5 times lower than Pb- ones. It represents a reduction of the disease of about 77% .

Table 4.5: Disease reduction (%) in *P. betae* infected (Pb+) plants regarding to the controls (Pb-). Reduction is calculated as 1 - (mean disease area Pb+/mean disease area Pb-)

.

0 dpi	9 dpi	12 dpi	14 dpi	16 dpi
0	98.5	97.2	89.64	77.11

4.2.4 Discussion

A reduction of CLS on sugar beet was clearly observed when plants were previously inoculated with *P. betae*. The calculated disease reduction at 16 dpi is similar to results obtained by Bargabus *et al.* (2002) (see tab. 4.4). In spite of diseased areas are not important at 9 dpi, the disease reduction at this time is the higher, with 99% less diseased area in Pb+

plants. In a large extent, it is due to the very high number of plants showing symptoms (15 plants) compared to the three ones in the Pb+ plants. Regarding to the dynamic of disease progress at each time, especially number of symptomatic plants and progress of Cercospora leaf spot leaf by leaf (data not shown), the inoculation of *P. betae* seems to result in a double effect. The infection by the *C. beticola* is delayed, and the progression rate was reduced. We hypothesize that these characteristics correspond to a response of systemic acquired resistance. The delay of infection could be explained by some events caused by SAR, such as cell walls thickening, while the progression of the fungi could be repressed by SAR associated metabolites acting inside the cells, e.g., chitinases or proteinases inhibitors (Vallad & Goodman, 2004; Van Loon *et al.*, 1998; Van Loon & Van Strien, 1999). Further histological and molecular studies should reveal the underlying mechanisms of this delay of infection. Moreover, it could be very interesting to assess a putative correlation in field between infection potential of soils in *P. betae* and impact of CLS. This field study could be very helpful in the understanding and evaluation of the benefit.

These results demonstrated that *P. betae* infection of sugar beet conferred a form of resistance of the plant to CLS. It is the first report of a putative effect of *P. betae* against a pathogen through natural plant defenses.

CHAPTER 5

GENERAL DISCUSSION

5.1 Metabolic and immunitary events

The aim of this thesis was to better understand the molecular interactions between *P. betae* and *B. vulgaris*. Thanks to the transcriptomic and defense ways analysis, we highlighted metabolic ways and biological processes involved in this interaction. In this part, we present a first model of molecular interactions between sugar beet and *P. betae*.

Interaction *P. betae*-sugar beet, a recapitulative scheme This paragraph presents events highlighted or hypothesized in this thesis concerning the defense interactions between *P. betae* and sugar beet (fig. 5.1). Some ESTs obtained during the infection, e.g. BvRamp, BvToc, BvTrx,... evidence that infection of sugar beet by *P. betae* induces ROS production and thus oxidative stress. Multiple causes can generate this stress. First, the ROS could be induced by the classical way, the PAMPs recognition by sugar beet. However, the disruption of the membrane by the protist Stächel could also be involved in ROS production. These compounds generating oxidative stress could be putatively produced as secondary metabolites by the plant, as defense compounds, or by the protist, in order to degrade parietal components. ROS are known to induce SAR, which in this case has been proven to reduce infection of

sugar beet by *Cercospora beticola*. The SAR and accompanying PR proteins, BvCys, BvPR10, BvPAL,... do not have an impact on *P. betae*, on the contrary of ISR. This last defense way reduces strongly the infection by *P. betae*.

Putative stress During the interaction between *P. betae* and sugar beet, plant defense ways are activated probably due to the stress caused by the presence of *P. betae*. Origins of the stress can be the oxidative stress due to the infection (see fig. 5.1), the stress due to the feeding requirements of the protist and, at last, the stress correlated to the massive presence of the protist in root cells.

As revealed in the transcriptome analysis of the *P. betae*-sugar beet interactions, several molecules with an homeostasis ability or enzymes active against oxidative stress are produced by both organisms. Indeed, two sugar beet transcripts were found coding for enzymes involved in this protection, thioredoxin and tocopherol cyclase. Moreover, plant ESTs coding for protection or homeostasis proteins were also found in infected sugar beets (unknown homeostasis protein, ribosome associated membrane protein). These ESTs were detected during the infection phase (3 to 24 hours post-infection) and the rest of the cycle (5 and 15 days post infection). Along the whole protist life cycle, the plant seems to react to an oxidative stress which could be associated to the SAR induction (see below). The results revealed that the parasite also suffered from oxidative stress: glutathione, a buffer molecule, is likely produced by the protist given the constitutive expression by *P. betae* of glutathione-S-transferase (GST) during its whole cycle (Kingsnorth *et al.*, 2003; Mutasa-Göttgens *et al.*, 2000). These results suggest that an oxidative stress, associated with reactive oxygen species (ROS) production, occurs during interaction between *P. betae* and sugar beet. ROS are common by-products of metabolic reactions, dangerous but scavenged under normal conditions. A troublemaker event can interfere with this equilibrium and induce an oxidative stress, which must be counteracted by the plant. These ROS have become, during evolution, key factors in plant defense responses (Heller & Tudzynski, 2011), principally in signal interaction processes. In the *P. betae*-sugar beet interaction, in addition to a first response of the plant to the infection, ROS could be directed against the parasite, directly or undirectly, through systemic defense

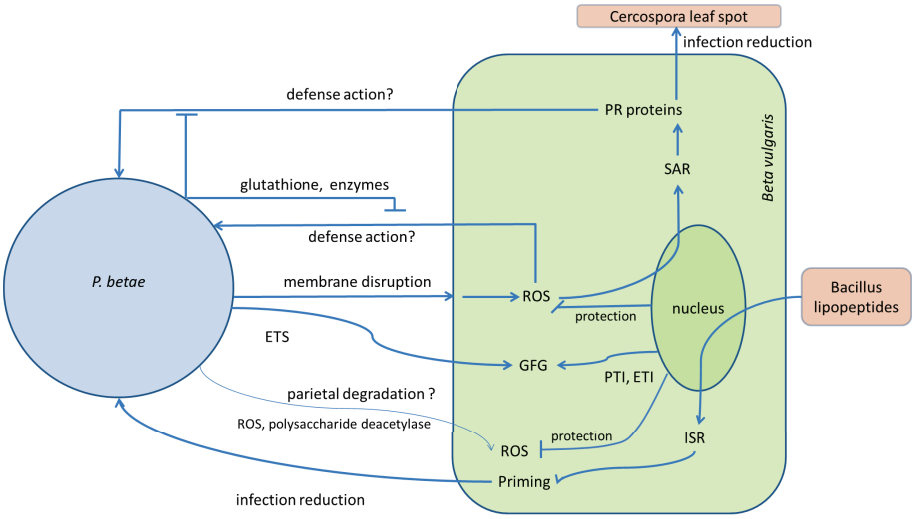

Fig. 5.1 : Recapitulative scheme of the metabolic events occurring during the infection of sugar beet by *P. betae*. During the injection of the protist cytoplasm inside the cell, the pattern recognition or disturbance of plasmic membrane by penetration induce ROS production. This ROS production is suggested by the presence of redox homeostasis proteins and of proteins confering protection against oxidative stress (BvHom, BvRamp, BvToc,...) (see section 3.1). This production of oxidative compounds can target directly *P. betae*, which protects itself with buffer molecules or enzymes. This protection is suggested by the presence of PbGST involved in the production of gluthatione. ROS also induce systemic resistance of the plant. PR proteins, PR-10, defensins, etc. revealed in the differential transcript analysis and resulting from SAR activation are neutralized by *P. betae* but are effective against pathogens such as *C. beticola* (see section 4.2), as shown in this thesis. Another way of defense, likely after recognition of specific protist proteins, is induced: it is part of the zig-zag of the gene to gene model (ETS, ETI, PTI). *Polymyxa betae* produces probably ROS, likely to digest more easily plant parietal components thanks to enzymes evidenced in our study, such as polysaccharide deacetylases (PbPode). Plant produces protection compounds against this stress too, with homeostasis proteins and enzymes actives against oxidative stress. Endly, membrane disturbance by *Bacillus* lipopeptides induces the other systemic way, the ISR. On the contrary of the SAR, this way is effective against *P. betae*, and reduces drastically infection by the protist (section 4.1)

way induction. A part of the oxidative stress of the plant could be also the result of *P. betae* ROS. These ROS could help the protist to colonize plant cells or to feed itself thanks to the plant cellulose degradation, as in the case of the fungus *Podospora anserina* (Brun *et al.*, 2009). The identification of polysaccharide deacetylases (PbPode) during whole cycle of *P. betae* suggests such parietal degradation. Homeostasis proteins or enzymes, such as thioredoxins, are known to confer protection against oxidative stress (Jamieson, 1998; Arner & Holgrem, 2000). The presence of ESTs encoding for these proteins, highlighted by our analysis, confirm that whatever the origin of this oxidative stress, the sugar beet can counter these agressive ROS. Parasites can also counter the plant ROS by producing enzymes like peroxidases or molecules acting as peroxide quenchers (mannitol derivatives e.g.)(Molina & Kahmann, 2007; Voegele *et al.*, 2005). This is the case of *P. betae* with the production of glutathione (presence of glutathione-S-transferase). The different origins of the ROS behind the protective response observed in our analysis are illustrated in the recapitulative scheme (fig. 5.1).

The differential transcript analysis revealed the production of two putative aquaporins (Bvaq and BvMip) when *P. betae* was present. These water transport proteins can be involved in parasite-plant interactions and considered as an adaptative response to the plant cell changes induced by infection (Maurel *et al.*, 2008). In this way, giant cells produced by the root-knot nematode were reported to be correlated with the production of an aquaporin ensuring the extensive delivery of water and solutes to the parasite (Opperman & Conkling, 1994). The production of aquaporins by sugar beet in response to *P. betae* infection could be associated to the same functions. After several cycles of multiplication of the protist in optimal conditions, up to 90% of cortical cells of sugar beet rootlets are infected. This presence could in fact induce a hydric stress and then induce production of specific aquaporins. The overexpression of these two water transporters could correspond to a stress due to feeding requirements and massive presence of the protist in the sugar beet roots.

Induction of systemic resistance ways The transcriptome and the gene expression profiling analysis revealed that some genes encoding for PR-proteins (BvCys, BvPR10, BvRip,. . .) were overexpressed. The PR-

protein produced by sugar beet reveals that SAR is induced by *P. betae*. This induction could be triggered by two putative different ways. The first way is the recognition of PAMPs by plant receptors. This recognition induces activation of Ca^{2+} channels and thus increase of Ca^{2+} concentration. This increase triggers a transcriptomic response and activation of NAPDH oxidase, which produces H_2O_2 (fig 5.2). Another possibility, recently suggested, is that a perturbation of the membrane, by the protist Stächel[1] e.g., could activate mechanically induced membrane channels, which could induce an unknown metabolic cascade to trigger plant defenses and thus ROS. Such induction mechanism was hypothesized on tobacco (Stanislas *et al.*, 2009), suggesting that a disruption in membranes leads to an activation of plant defenses. This hypothesis is supported by the discovery that activity of membrane proteins may be more dependent on the surrounding lipid organization than previously estimated. Henry *et al.* (2011) evidenced such kind of membrane disruption in plant defense induction involving *Bacillus* lipopeptides.

Plasmodium, the immunitary active stage The differential transcript analysis, as well as the gene expression profiling, showed that most of defense transcripts were overexpressed between one and five days after inoculation of the plant roots by *P. betae* zoospores. During this period, the protist invades the plant cell and the plasmodium grows in the cell. The protist enters in a vegetative phase. During this time, a membrane is synthesized, and numerous nuclei are produced inside the plasmodium. The development of plasmodium is putatively highly dependent on the two evidenced genes, PbPro and PbvWf, respectively encoding for profilin and von Willebrand factor domain containing protein. The transcripts for these two proteins were overexpressed during the growth of the plasmodium. This vegetative phase is probably the most costly phase for the sugar beet because *P. betae* invades host cells and produces multiple nuclei in each sugar beet cell. The needs of the protist for energy and nucleic acids are likely important. Moreover, at the plasmodial stage, the membrane is under formation and no cell wall is present (Barr & Asher, 1996; Braselton, 1995) on the contrary to zoosporangia and sporosori. The plasmodium is therefore a weak

[1] "Needle" which helps the zoospores to pierce the plant cell wall.

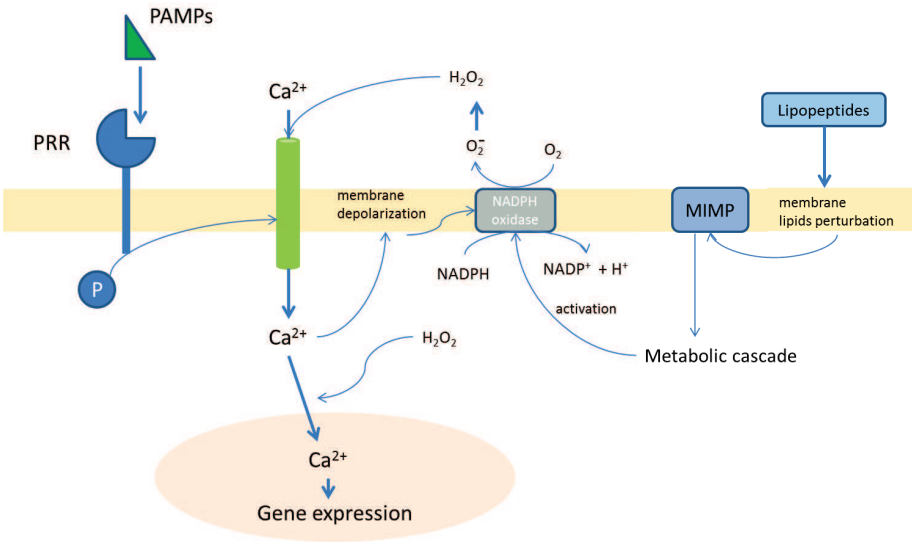

Fig. 5.2 : Two possible ways of early events which can lead to plant defense responses. The first occurs after recognition of PAMPs or MAMPs. The pattern recognition receptor (PRR), a kinase, activates a calcium channel. The accumulation of calcium inside the cell depolarizes the membrane and leads to the activation of NADPH oxidase, which produces oxygen radicals, rapidly transformed in H_2O_2. With the common action of Ca^{2+} and ROS, the plant defenses are activated. The other way to activate plant defenses is to disturb the membrane as lipopeptides do. The disturbance of the membrane can activate mechanically-induced membrane proteins (MIMP), which induces a metabolic cascade leading to the activation of NADPH oxidase. Adapted from Jourdan *et al.* (2008) and Henry *et al.* (2011).

stage in sugar beet root cells and a great energy consumer. Most of the analyzed gene expressions involved in plant defenses were found to be expressed at this stage[2]. But these defenses are not supposed to be effective against the protist which continues its development and spread inside the plant cells. Interestingly, a gene silencing machinery seemed to be set up, as suggested by the expression of a gene encoding for RNA dependent RNA polymerase, involved in gene silencing (BvRpolR). As

[2]Eight of the eleven analyzed gene expressions showed overexpression between one and five days after infection.

for the evidenced overexpressed proteasome (BvProt), sugar beet seemed to try to take advantage of the direct contact with protist cytoplasm or membrane.

Zoosporangium and sporosorus, more silencious stages On the contrary of the plasmodial phase, only two (cystein-rich protein [BvCys] and protease inhibitor [BvPin]) of the eleven analyzed genes were overexpressed at the sporangial and sporogenic phases (15 days post-infection). As the dynamic of gene expression, the ESTs identification suggested than plant defenses were less active after five days of infection: nine ESTs were identified as involved in defense or stress response between five and fifteen dpi while seventeen were identified in the first 24h. The metabolic pathways occurring during sporangial and sporogenic phases are different from those occurring during the plasmodial stage. Nuclei are enclosed during both sporangial and sporogenic phases, but in different goals and cellular components. During the zoosporangial stage, single membranes are formed in zoosporangial lobes in order to produce zoospores. In sporosori, multiple layers are deposited around the membrane to form survival spores (Barr & Asher, 1996). These processes are energy consumers but these stages could nevertheless represent a lesser cost for the sugar beet than the plasmodial phase. Indeed, Mithen & Magrath (1992) showed that starch granules in *Plasmodiophora brassicae* cells (evidenced by Buczacki & Moxham (1979)) could be integrated by the protist during plasmodial phase. Moreover, Barr & Asher (1996) showed that the same structures, enclosed in vacuoles, were present in *P. betae*. By this way, the plasmodiophorids could store energy by hijacking plant energy stocks during their plasmodial phase. The sporangial and sporogenic phases taken apart from the plasmodial phase could be less costly for the sugar beet, energetically speaking. Moreover, during sporangial and sporogenic phases, a cell wall is formed, which is not the case in the plasmodial phase. This cell wall likely reduces exchanges and interactions between both organisms. Both the reduction of parasitism costs or effects on the host and the cell wall formation could explain the less active defenses of the plant.

5.2 Kind of parasitism of *Polymyxa betae*

Despite the fact that *P. betae* can infect a great part of sugar beet root cells, its infection remains asymptomatic. In this view, an interesting question related to the protist is which kind of parasitism it exerts on sugar beet. As described by Combes (2010), the three kinds of parasitisms are the pathogenic parasitism, the commensalism or the mutualism. These kinds of parasitisms are determined by a cost-benefit balance, the pathogenic parasitism being more costly than benefic, the commensalism neutral and the mutualism more benefic than costly. In this section, we attempt to determine the kind of parasitism of *P. betae* exerted on sugar beet.

Resources allocations, parasitism and defenses The analysis of the plant defense responses showed that most of the energy devoted to the defense was spent during the plasmodial phase, while, during sporangial and sporogenic phases, defenses seemed to be reduced: only two genes were overexpressed (BvCys and BvPin), while the number of ESTs related to defense and stress decreased from 17 (3-24h) to 9 (5-15 days). Why does the plant reduce its investment in defense after fifteen days? In a living cell, energetical resources can be considered as a stack of coins (fig. 5.3). Each coin can be allocated to a specific function. In case of parasitism, the plant defenses have a cost, as the parasite hijacks energy and raw materials. As long as the cost of the parasite is higher than defense costs, defense state would be maintained in the plant. In the first days following the infection, the plant attempts to stop the protist progression. The needs of protist are important, as well as the investments in defense of the plant. Fifteen days after infection, the first wave of infection is finished and the protist is getting ready for the remainder of the season (zoosporangium) and for long-time survival (sporosori). After the strong nucleus multiplication of plasmodium, zoosporangium and sporosori are more separated from the plant cell by cell walls, reducing interactions. Sporosori, the survival structures, are less active. For zoosporangium, most of the energy has probably been accumulated before: the stage is more a stage of maturation where single nuclei are enclosed with energy stocks allowing the short survival of the zoospore

in a single membrane synthesized de novo (Barr & Asher, 1996). These synthesis is likely the highest energetic cost of the stage.

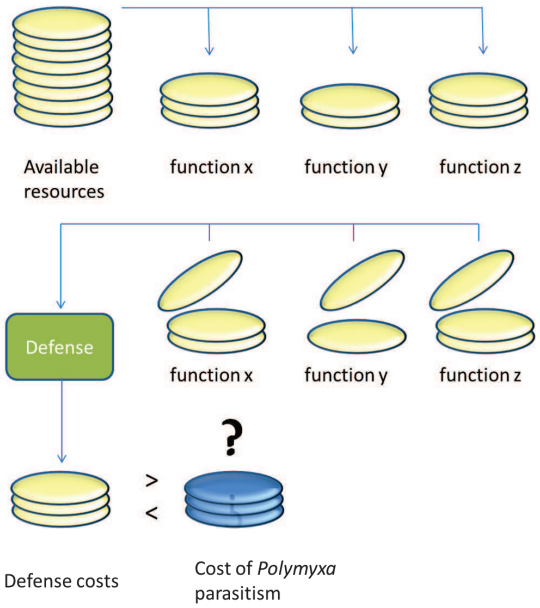

Fig. 5.3 : Resources allocation. The plant disposes of limited energetical and raw material resources, which are allocated to specific functions (x, y, z). When a parasite infects the plant, it consumes a part of these stocks. But the defense responses also represent a cost. These defense responses will be advantageous to the plant if they are less costly than parasite costs. If the contrary occurs, plant defenses will be phased out. Adapted from Combes (2010).

Cost-benefit balance of the interaction *P. betae* - *B. vulgaris*
As discussed previously, after penetration of *P. betae* into the cell and during the plasmodial phase, *P. betae* uses energetical resources and nutrients from the sugar beet. At this moment, plant invests resources in defenses. We hypothesize that five days after infection, the plant defenses are less costly to the plant than the parasitism costs. The defenses are directed against the protist and SAR is also induced, as suggested by PR-proteins. Fifteen day after infection, as previously discussed, the cost of parasitism is probably very low, if stocks are effectively done dur-

ing the plasmodial stage. Moreover, it was shown that the SAR induced by *P. betae* infection was effective against *C. beticola*, even fifteen days after infection. This protection constitutes thus an advantage for the plant, and could also putatively explain the loss of active mechanisms of defense against *P. betae*. After fifteen days, the benefit is established (SAR) while the costs seem to decrease.

Kind of parasitism of *P. betae* Even if *P. betae* induces plant defenses, and given the likely decreasing cost for the plant along the life cycle of the protist, the reduction of foliar disease such as Cercospora leaf spot is a clear benefit (fig. 5.1). Moreover, in this case, this benefit is not counterbalanced by clear pathogenic symptoms, which is the case with other plasmodiophorids as *P. brassicae* or *S. subterranea*. In this thesis, we evidenced that priming induced by ISR reduced severely *P. betae* infection (fig. 5.1). Sugar beet gets thus the weapons to counter the parasitic development of *P. betae*, but in absence of PGPR, these arms are not produced under natural conditions. As developed in the general introduction, all parasitic systems begin with a pathogenic relationship. After, this relationship can evolve to commensalism or mutualism, by deletion of pathogenicity genes, e.g. (Combes, 1995). Even in the case of an evolution to a relationship of commensalism or mutualism, several studies showed that the mechanisms underlying the presence of an organism in the plant, such as defense mechanisms, remain. It is the case of mycorrhizas, e.g., known as mutualists but inducing systemic resistance (Pozo *et al.*, 2009; Whipps, 2004). *P. betae* is not a symbiont because Pb+ plants do not show a higher fitness than healthy ones. However, given the absence of symptoms, the apparent reduction of defenses after fifteen days and the benefit due to the induction of systemic resistance, we consider this protist as a commensal maybe evolving to mutualist, following conditions and presence of pathogens as *C. beticola*. Nevertheless, *P. betae* does not always infect sugar beet alone. The protist is sometimes carrier of the BNYVV, which changes the cost-benefit balance...

The extended parasitism, after the extended phenotype Breeders behaviour follows the logic of evolution. Evolution selects new host genes to counter the pathogen progression. Breeders accelerate evolu-

tion by selecting plant harboring resistance genes to suppress pathogen infections. Up to now, no resistance against *P. betae* was found in *B. vulgaris*. This reinforces the hypothesis of commensalism (or light mutualism) concerning this interaction. Resistance genes against *P. betae* were found in wild *Beta* species (Barr *et al.*, 1995) and introgressed in sugar beet varieties but they were never used in agriculture due to the decrease of yield associated with these genotypes. In the complex pathosystem, *P. betae* is the vector of BNYVV, one of the most damaging pathogens of sugar beet. This virus, after being a sugar beet pathogen, could be considered as the parasitic pathogen of the interaction *P. betae*-sugar beet, an extended parasite of both hosts, using their efficient interaction. In natural conditions, an allele of resistance against the vector should be selected by evolution. But in agroecosystems, selection is under the breeder's control. If until recently, only some authors suggested to control rhizomania through a resistance against *P. betae* (Asher & Barr, 1993), this solution could appear to be more interesting nowadays for breeders, given the resistance breakdowns observed with the alleles Rz1 and Rz2 (Acosta-Leal *et al.*, 2007; Bornemann & Varrelmann, 2011). Therefore the pathogenicity of BNYVV could be considered by the breeders to be "extended" to *P. betae*, since it contributes to the viral spread and transmission. This behaviour boils down to consider virus and vector as one organism, hence the term "extended parasitism" in parallel to the extended phenotype of Dawkins (1982)[3]. The extended parasitism model appears to be particularly well adapted to characterize *Polymyxa* spp.-plant interactions. Indeed, beside the complex *P. betae*-BNYVV interaction, *Polymyxa* genus is associated to the transmission of numerous viruses belonging to four genera. This was shown by Dieryck *et al.* (2011), who evidenced a more pronounced geographical coevolution between *Polymyxa graminis* f. spp., plants and viruses isolates transmitted. We demonstrated in our work that in absence of virus, *P. betae* provides likely benefits to the plants. By controlling the protist, the benefits could be lost and resurgence of other diseases observed. To break this equilibrium protist-plant could thus generate other problems in sugar beet crops. Breeders have to handle this new resistance with

[3]Dawkins developed the concept of extended phenotype for phenotypes present only when two organisms interact. An example is the crown gall, a phenotype developed due to the interaction of *Agrobacterium tumefaciens* and plants.

care and costs of a control of *P. betae* in the management of rhizomania have to be considered regarding other sugar beet diseases. As infection by *P. betae* ensures the induction of SAR, an interesting integrated control could be the combination of varieties tolerant and susceptible to *P. betae* but tolerant to virus, allowing to decrease the virus content while benefits of the protist are conserved: even in the tolerant case, the primary stages of infection could occur and induce SAR. The association of different varieties in a same field is considered as a useful tool of integrated crop management, considerably reducing selection pressures in the field (Keesing *et al.*, 2006). This way the extended parasitism could be controlled.

GENERAL CONCLUSION

The main objective of this thesis was to better understand the molecular interactions between *P. betae* and sugar beet. After the development of two new study models, we highlighted some genes involved in sugar beet - *P. betae* interactions using a transcriptomic analysis and by quantifying the expression of some of these genes along the life cycle of the protist in its host. Endly, as markers of systemic resistance ways of the plant were overexpressed during the interaction *P. betae*-sugar beet, we investigated the interactions between *P. betae* and plant defense ways when *P. betae* was upstream and downstream.

Establishment of new studies models

Two new models to study plant-*P. betae* interactions were developed in a first part of the thesis. First, we found compatible interactions between *A. thaliana* ecotypes and *P. betae*. This model will be helpful for further genomic studies: segregation between DNA sequences from plant and protist will be easier. Moreover, *A. thaliana* provides transcriptomics tools as microarrays. In the manner of McGrann *et al.* (2007), it could be useful to compare the expression of plant genes depending on the degree of compatibility between *P. betae* and plants. Transcriptomic studies on *P. betae*-*A. thaliana* interactions could also be compared to those done with sugar beet in this thesis. The other study model developed

in this thesis is the dual culture of *P. betae* on sugar beet hairy roots. This model is the first to establish a plasmodiophorid culture in a liquid media. As opposed to other plasmodiophorids dual cultures (*Plasmodiophora brassicae* (Mugnier, 1987) and *Spongospora subterranea* (Qu & Christ, 2007)), this model enables the swimming of secondary zoospores and secondary infection of the hairy roots. It could be used as a novel strategy for a continuous production of zoospores.

Transcriptomic analysis: the molecular dialogue between *P. betae* and sugar beet

In the second part of the thesis, some genes overexpressed during the interaction between *P. betae* and *B. vulgaris* were evidenced using a suppressive subtractive hybridization. The dynamic of expression of some genes was followed by qRT-PCR along the protist life cycle. This analysis revealed a rapid defense response from the plant with presence of transcripts corresponding to SAR-dependent proteins, such as PR proteins, even three hours after *P. betae* inoculation. Most of sugar beet defense genes were overexpressed between one and five days after *P. betae* inoculation. This activity is explained in this work by the fact that plasmodium is the most active life stage in the protist life cycle. Indeed, during this stage, nuclei are multiplied in mass in order to prepare the future life stages, i.e., zoosporangia and sporosori. As previously discussed, the nutrient storage of plasmodiophorids is likely done during the plasmodial phase energy by hijacking starch granules from the host cells. By this way, sporosori and zoosporangia could be "neutral" for the host. This could explain the decrease of defense responses of the plant fifteen days after *P. betae* inoculation. For *P. betae*, the tested genes showed also interesting profiles. The results highlighted overexpression of two genes probably correlated with the infection phase of the protist: profilin, a protein associated with the cytoskeleton and crucial in the infection phase of *Toxoplasma gondii*, another protist, and a protein containing a von Willebrand factor domain, similar to one protein produced by *Plasmodium falciparum* and necessary for the infection of its host. Other *P. betae* genes seemed to be expressed constitutively. Nevertheless, the EST encoding putatively for galactose-binding lectin, prob-

ably important in cell recognition during infection, is repressed when the life stage zoospore is absent (five days). The *P. betae* polysaccharide deacetylase sequenced in this study could be involved in parietal degradation and thus in cell-to-cell movement, as suggested by Bulman *et al.* (2006) for the interaction *P. brassicae-A. thaliana*. For *P. betae*-sugar beet interaction, the RNA corresponding to this protein is overexpressed after 24 hours of infection. This could correspond to a greater activity of movement 24 hours after inoculation. Moreover, the putative higher degradation of parietal components observed after 24 h and, to a lesser extent after five days, strengthens the hypothesis of a great cost for the host of the plasmodial phase in comparison to zoosporangia and sporosori. Endly, the G protein, a transmembrane signal protein, seemed to be overproduced after five days when most of plasmodial membranes are produced. This transcriptomic analysis enabled to discover about seventy new parts of *P. betae* genes but also to better understand interactions between the protist and sugar beet. Thanks to this analysis, we highlighted that the most "immunitary active" stage was the plasmodium, while plant defenses are less expressed during further life stages of the protist. This decrease in defense corresponds likely to a decrease in the costs for the host of the sporangial and sporogenic phases of *P. betae*.

P. betae up- and downstream of plant defenses: what is going on there?

During the transcriptomic analysis, ESTs coding for several PR proteins were sequenced. They revealed that *P. betae* induced the systemic resistance ways of the plant. In order to understand what are the real interactions between *P. betae* and sugar beet resistance ways and their impacts, two experiments were conducted. During the first experiment, *P. betae* was put downstream the plant systemic ways: ISR was induced by *Bacillus* lipopeptides and thereafter sugar beet plants were challenged with *P. betae*. This treatment reduced *P. betae* infection up to about 90%. These results evidenced that sugar beet has the weapons to reduce *P. betae* but that they are not activated in absence of PGPR. Moreover, the effect of lipopeptides treatment on *P. betae* could have a positive im-

pact in an integrated management of rhizomania. This treatment limits vector infection and could be combined with the classical control strategy as the use of varieties tolerant to BNYVV. Nevertheless, as SAR was evidenced to be induced by *P. betae* presence by transcriptomics analysis, we tested *P. betae* upstream the SAR, as elicitor. After inoculation of *P. betae*, sugar beet leaves were challenged with *Cercospora beticola*, and dynamics of CLS disease were analyzed. *P. betae*-infected plants showed significant reduction of symptoms, in the same order of magnitude (about 70% of disease reduction) than in previous studies using chemical elicitors or PGPR (Bargabus *et al.*, 2002). This observation revealed the advantage conferred by *P. betae* to the plant. A field approach aiming to determine if a correlation between infectious potential of *P. betae* and CLS incidence exists would be useful to determine the real benefit for the plant.

P. betae, a commensal parasite

Given the results of these thesis, the most likely kind of parasitism exerted by *P. betae* alone is the commensalism: even if the parasite has costs, principally to feed, but also because of the stress, e.g., as a protection agains ROS. However, these costs seem to be relatively limited: the absence of symptoms and effects on plant physiology or root weight (Moreau, J-S., Desoignies N. Legrève A., unpublished) argue in this sense. Indeed, the most costly stage is probably the plasmodium, the first stage of the protist life cycle. Then, plant defenses are decreasing which probably means that, at this time, defense costs are higher than the cost of *P. betae* parasitism. On the other hand, we found a "hidden benefit" of *P. betae*. Indeed the protist induces a systemic resistance effective against important pathogens like *C. beticola*. This benefit can balance the nutrition and stress costs occasioned by *P. betae* and makes it a commensal.

This "peaceful" interaction of commensalism can be endangered by the presence of the BNYVV. The ability of *P. betae* to transmit this virus is a supplementary cost. The commensal interaction of sugar beet and *P. betae* is thus parasited by the virus. The question of relevance to control rhizomania through resistance against the vector still remains unresolved. Indeed, controlling this protist by varietal resistance or siRNA

strategies could help to control BNYVV but might also reinforce the impact of other diseases, such as *C. beticola*, in sugar beet fields. The solution could be to combine varieties tolerant to *P. betae*, in order to reduce the pressure of BNYVV, and varieties susceptible to *P. betae*, allowing to preserve the benefits provided by this protist.

Perspectives

ISR action against *P. betae* A first complementary work to our study concerns the understanding of the protective effect of *Bacillus* lipopeptides against *P. betae* infection. If the underlying mechanism - the induced systemic resistance - is known (Nihorimbere *et al.*, 2012), the determinant molecules or events which reduce effectively *P. betae* progression remain unclear. The identification of these determinants will provide an important advance in the understanding of interaction mechanisms and, in particular, it will provide insights for new strategies of rhizomania control. Moreover, application in fields have to be tested. The coating of seeds by lipopeptides appears to be a practical way to induce the systemic resistance early in the plant development.

Bypass of the extended parasitism Lauber *et al.* (1998) were the first to use a replicon, RNA derived from BNYVV to study gene functions. In the same way, this technique could be used to study functions of the discovered *P. betae* genes, and maybe to silence some *P. betae* gene products by producing si-RNAs. The silencing of the profilin and of the von Willebrand factor domain containing protein - two proteins suspected to play an important role in cell colonization and after the first hours of infection - could be an interesting way to limit *P. betae* infection. Another possibility would be to use the replicon to overexpress defense genes of sugar beet inside the protist. The close relationship between BNYVV and *P. betae* could hence be hijacked to suppress the two organisms, the "extended parasite". Varieties using the si-RNA technology, could be developed or tested in an integated pest management view.

Protective effect of *P. betae* against other pathogens The SAR induced by *P. betae* was evidenced to reduce infection of sugar beet leaves by *C. beticola*. It would be interesting to test this protective effect of *P. betae* against other pathogens. Sugar beet elicited by *Trichoderma harzanium* showed lower levels of *Rhizoctonia solani* (Anees *et al.*, 2010). If no other studies were done in sugar beet pathosystems, some infections of pathogens close to sugar beet pathogens are reduced by systemic resistances. Rust causal agents and nematodes are also reduced by SAR (Molinari & Baser, 2010; Sillero *et al.*, 2012). Sugar beet rust (*Uromyces betae*) and the sugar beet nematode (*Heterodera schachtii*) could also be challenged with *P. betae*-infected and non infected sugar beets, in order to check if the protective effect of *P. betae* can be generalized to sugar beet pathogens. This benefit could consolidate the fact that commensalism, or even slight mutualism is the kind of parasitism of *P. betae*.

Genome of *P. betae* New ESTs of *P. betae* were sequenced. A first way to enlarge the genome knowledge of this protist will be the sequencing of genes related to these ESTs, with a 3'- and 5'-rapid amplification of cDNA ends (RACE). Moreover, the new models developed in this study will allow the sequencing of new genes by sequencing directly pure zoospores obtained in dual culture or by differential transcripts analysis techniques on *A. thaliana*. The discovery of new genes of *P. betae*, putatively involved in key stages of the protist or in the viral transmission, could be interesting for new strategies of rhizomania control based on RNA silencing, example given.

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APPENDIX A

BETA VULGARIS SEQUENCES USED FOR SEQUENCES SEGREGATION (SECTION 2.2)

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APPENDIX B

CERCOZOA SEQUENCES USED FOR SEQUENCES SEGREGATION (SECTION 2.2)

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APPENDIX C

BIOLOGICAL PROCESSES OF BETA VULGARIS ESTS FOUND IN THE SECTION 2.2

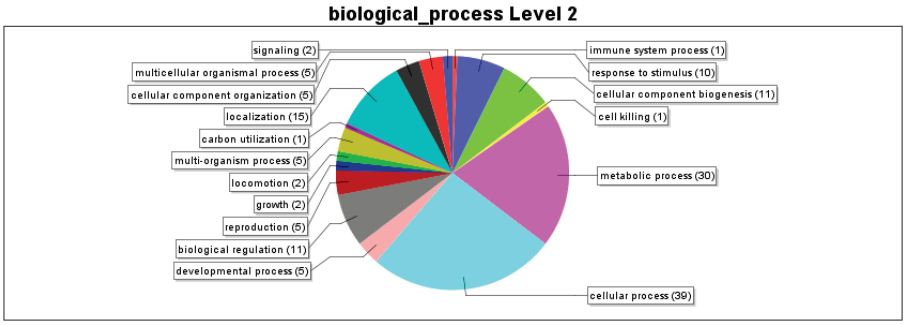

Fig. 1 : Biological processes of the sugar beet ESTs found 3 and 24 hours after *P. betae* infection

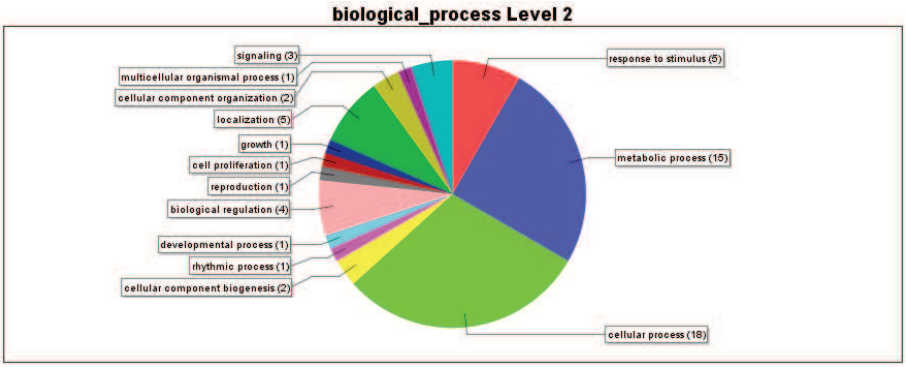

Fig. 2 : Biological processes of the sugar beet ESTs found five and fifteen days after *P. betae* inoculation.