Molecular aspects of early microbial infection in *Medicago truncatula* Gaertn.

Von der Naturwissenschaftlichen Fakultät
der Gottfried Wilhelm Leibniz Universität Hannover
zur Erlangung des Grades
Doktor der Naturwissenschaften
Dr. rer. nat.

genehmigte Dissertation
von
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Tag der Promotion:	20th December 2013

Abstract

This thesis aims to provide a profound understanding of the molecular mechanisms underlying symbiotic and pathogenic interactions in legumes. Legumes (Fabaceae) are unique in their ability to form elaborate endosymbiosis with nitrogen-fixing rhizobial bacteria, but also interact with other microbes including pathogens. The model legume *Medicago truncatula* Gaertn. is used in this study as a host for the two types of interactions. Symbiotic interactions were achieved with rhizobia bacteria *Sinorrhizobium meliloti* and the arbuscular mycorrhizal fungi *Glomus intraradices*, while the root rot oomycete *Aphanomyces euteiches* was used for pathogenic interactions. The study applies molecular and proteomic approaches to systematically identify and characterize genes and proteins involved during the early phases of symbiotic and pathogenic interactions.

A study on the role of *M. truncatula* MtROP9 via an RNA interference silencing approach is presented. ROP (Rho of plants) are plant-specific small GTPases that cycle between active (GTP-bound) and inactive (GDP-bound) conformations, functioning as molecular switches within elementary signal transduction pathways. ROPs form key regulatory elements for reactive oxygen species (ROS) generation in plant cells via activation of NADPH oxidase homologue termed respiratory burst oxidase homologue (RBOH). Various physiological and morphological changes in MtROP9i plants are shown including growth-reduced phenotype and strongly deformed and reduced number of nodules. The oxidative burst measurements from the infected roots revealed neither ROS production nor expression of MtROP9 and MtRBOH genes. Proteome analyses via two-dimensional differential gel electrophoresis revealed suppressed induction of antioxidative compounds in MtROP9i roots.

In another project, an analysis of the *M. truncatula* mitochondrial proteome from root cell suspension cultures was carried out. Data are displayed via the GelMap portal (www.gelmap.de). Different protein complexes were identified alongside that of *Arabidopsis thaliana*.

An analysis of the temporal proteome response of *M. truncatula* roots to microbial infections under conditions of significantly suppressed potential for ROS synthesis is presented in the final section of this thesis. Usage of the new legume database LegProt coupled with sensitive MS analysis resulted in high protein identification rates. Data are presented via a novel heatmap module implemented into GelMap software tool.

Keywords: *Medicago truncatula*, pathogenic interactions, symbiotic interactions

Zusammenfassung

Diese Arbeit wurde angefertigt um ein tieferes Verständnis der molekularen Mechanismen von symbiotischen und pathogenen Interaktionen in Hülsenfrüchten (Leguminosen) zu erlangen. Hülsenfrüchte (Fabaceae) besitzen die einzigartige Fähigkeit, eine hochspezialisierte Endosymbiose mit Stickstofffixierenden Knöllchenbakterien (Rhizobiales) auszuprägen; zudem interagieren sie mit einer Vielzahl anderer Mikroben wie beispielweise Krankheitserreger. In dieser Studie wurde die Modellpflanze *Medicago truncatula* Gaertn. als Wirtpflanze für die beiden Arten mikrobieller Assoziationen verwendet. Die symbiotischen Wechselwirkungen wurden anhand von Infektionen mit dem Knöllchenbakterium *Sinorrhizobium meliloti* und dem arbuskulären Mykorrhiza-Pilz *Glomus intraradices* in *M. truncatula* untersucht, wohingegen der Oomyzet *Aphanomyces euteiches* für die Untersuchung von pathogenen Wechselwirkungen verwendet wurde. Diese Studie hat sich zum Ziel gesetzt, molekulare und proteomische Ansätze zu verwenden, um Gene und Proteine, die in den frühen Phasen an der Etablierung der symbiotischen und pathogenen Wechselwirkungen beteiligt sind, zu identifizieren und zu charakterisieren.

In dieser Arbeit wurde die Rolle von *M. truncatula* MtROP9 über einen RNA-Interferenz Ansatz untersucht. ROP-Proteine („*rho proteins of plants*“) sind pflanzenspezifische GTPasen, die als molekulare Schalter in Signaltransduktionswegen dienen, indem sie zwischen einer aktiven Konformation (GTP-gebunden) und einer inaktiven Konformation (GDP-gebunden) wechseln. ROPs sind regulatorische Schlüsselelemente für die Generierung von Reaktiven Sauerstoffspezies (ROS) in den Pflanzenzellen über die Aktivierung von NADPH Oxidase Homologen, RBOH genannt („*respiratory burst oxidase homologue*“). Es konnten verschiedene physiologische und morphologische Veränderungen in den transgenen Pflanzen (MtROP9i) beobachtet werden, wie ein im Wachstum gehemmter Phänotyp, sowie das Auftreten stark verformter Wurzelknöllchen in deutlich reduzierter Anzahl. Die Messungen des „*oxidative burst*“ in infizierten MtROP9i Wurzeln zeigte weder eine ROS-Produktion, noch konnte eine Expression von MtROP9 und MtRBOH Genen gemessen werden. Experimente mittels zweidimensionaler differentieller Gelelektrophorese (2D-DIGE) zeigten eine unterdrückte Induktion von Proteinen mit antioxidativer Funktion in den MtROP9i Wurzeln. In einer weiteren Studie wurde das *M. truncatula* mitochondriale Proteom von Zellsuspensionskulturen analysiert. Die Daten wurden in die GelMap Software (www.gelmap.de) implementiert. Verschiedene mitochondriale Proteinkomplexe wurden mit denen aus *Arabidopsis thaliana* verglichen. Eine Analyse des temporären Proteoms während der Infektion von *M. truncatula* MtROP9i-Wurzeln mit den genannten Mikroorganismen wird im lezten Abschnitt dargestellt. Mit Hilfe verbesserter MS-Analysen in Verbindung mit neuen Leguminosen-Proteindatenbanken („*LegProt*“) konnte eine Vielzahl von Proteinen identifiziert werden, die mittles einer neuartigen „*heatmap-GelMap*“ dargestellt wurde.

Schlagwort : *Medicago truncatula*, pathogene Interaktionen, symbiotische Interaktionen

Contents

Abbreviations

AMF	arbuscular mycorrhizal fungi
CcaMK	calcium calmodulin dependent kinase
DMI	does not make infection
DNA	deoxyribonucleic acid
DOI	digital object identifier
ENOD	early nodulin
ERN	ethylene response factor for nodulation
GTP	guanosine triphosphate
HR	hypersensitive response
IPD	interacting protein of DMI
LCOs	lipochitooligosaccharides
Lj	*Lotus japonicus*
LRR	leucine rich receptor
LYR	leucine/tyrosine/arginine
LysM	Lysin motif
MAP	mitogen activated protein
Mbp	Mega base pair
Mt	*Medicago truncatula*
MtROP	*Medicago truncatula* rho of plants
Myc	mycorrhiza
NADPH	nicotinamide adenine dinucleotide phosphate homologue
Nepl	neprilysin-like protease
NFP	nodulation factor protein
NFs	nodulation factors
NORK	nodulation receptor kinase
NUP	nuclear pore protein
PAMPS	pathogen-associated molecular patterns

PCD	programmed cell death
PcF	*Phytophthora cactorum*-fragaria
PR	pathogenesis-related
R	resistance
Rac1	Ras-related C3 botulinum toxin substrate 1
RBOH	respiratory burst oxidase homologue
RL	rhizobia-legume
RLK/ RLP	receptor-like kinases/proteins
RNA	ribonucleic acid
RNAi	ribonucleic acid-interferred
ROS	reactive oxygen species
RT-PCR	reverse transcriptase polymerase chain reaction
SOD	superoxide dismutase
SYMRK	symbiosis receptor-like kinase
WGD	whole-genome duplication

CHAPTER 1

General introduction

Plants co-exist with microbes present in their environment in constant interactions. The mechanism by which they interact, associate and differentiate within each other has been at the forefront of any scientific inquiry. Initiation of a plant-microbe interface involves signalling molecules or factors perception from a potential microbe, triggering a flurry of molecular events that constitutes host's protein networks. Legumes (Fabaceae) which form a versatile and inexpensive protein source are at the core of this research. Their unique ability to carry out an elaborate endosymbiotic nitrogen fixation with rhizobia bacteria and interactions with broad array of other microbes foresaw their nomination as organisms of choice for studying plant-microbe interactions (Colditz and Braun, 2010). The legume crop species exhibit rather large and complicated genomes which render application of available molecular tools unsuccessful. Therefore, the two model plants *Medicago truncatula* Gaertn. (www.medicago.org) and *Lotus japonicus* L. (hwww.plantgdb.org/LjGDB) were selected, whose genomic tools have been established during the last two decades qualifying them as excellent platforms for genomic and proteomic research in legumes (Udvardi et al., 2005; Young and Udvardi, 2008). In *M. truncatula*, a draft sequence of the euchromatin was published recently capturing ca. 94% of all genes (Young et al., 2011), while the genome sequencing of *L. japonicus* was already completed in Japan in the past years (Cannon et al., 2005; Sato et al., 2008). These developments on nucleotide sequences have sparked knowledge on putative players in the scenario of plant-microbe interactions identified as proteins, of which constitutes the structural element of any organism. So far, substantial molecular, genetic, genomics, proteomics and metabolomics resources have been developed for these legume species to facilitate advances in the study of legume biology. Current progress in the area of proteomics also, has relied heavily on these genomic data and the development of mass spectrometry for sensitive, selective and high-throughput studies (Colditz and Braun 2010; Lee et al., 2013). Therefore, this introductory chapter aims to provide a background of plant-microbe interactions focusing on symbiotic and pathogenic interactions in legumes as well as presenting recent progress in this field of research.

1.1 Biology of plant-microbe interactions

Microbial infections in plants

The majority of organisms interacting with plants may be of bacterial, fungal or viral origin, nematodes, challenges by invertebrates, exposure to abiotic stresses as well as mechanical

wounding (Hammerschmidt et al., 2001). Owing to their sessile lifestyle, lack of mobile defender cells and a somatic adaptive immune system like in animals, they have evolved numerous adaptations to enable them cope with these unavoidable abiotic and biotic alterations and stresses. They rely mainly on their cellular innate defense systems as well as molecular signals that emanates during microbial infection (Gisholm et al., 2006; Jones et al., 2006). Majority of these microbes may exist as endophytes, implying that they cause no disease or pathogenicity which results in a disease infection (Kogel et al., 2006). In the course of evolutionary period, endophytes are considered to have transformed to establishing commensalistic or mutualistic associations in plants. A good example exists in the *Rhizobium*-plant symbiosis known to assume the primitive, intermediate and advanced stages of selective evolution. In this case, genes responsible for microbial interactions increased over time coupled with changes in strategies for niche occupation, with the most advanced symbionts ear-marked with exhibiting the highest degree of host selectivity and mutual recognition (Djordjevic et al., 1987). The commensalistic relationships are regarded as simple in contrast to the advanced complex *Rizobium*-plant interactions. Mutual recognition and molecular exchanges are predicted to play no role in establishment of commensal association and in this case, many rhizobia bacteria simply colonize the root surface and attach to the root epidermal cells including the root hair cells with no effect to the host (Djordjevic et al., 1987).

In a classical beneficial association, a microbe colonizes the host tissue and enters into a mutualistic association with the host plant. Some notable examples are those existing between legumes and the nitrogen-fixing rhizobia bacteria as well as the arbuscular mycorrhizal fungi (AMF). These microbes thrive as obligate symbionts in the host's roots, establishing symbiotic association, a relationship from which both the partners equally benefit. The outcome of rhizobia-legume symbiosis is nitrogen fixation into utilizable form (ammonia) by the host plant and in exchange, the rhizobia bacteria acquires the photosynthetic carbon. In AMF associations, the host acquires macronutrients such as phosphorus and nitrogen, as well as most likely an array of micronutrients, in exchange for plant-fixed carbon (Oldroyd et al., 2006; Finlay RD, 2008; Colditz and Braun, 2010). In contrast, during pathogenic interactions, microbes may exist as necrotrophs where they kill the invaded host tissue and utilize it for nutrients resource, or biotrophs by deriving its nutrients from a living tissue. Others still exist between these two groups, the so-called hemibiotrophs that once infecting, they proliferate at the infection site and spread some distance preceding onset of tissue damage (Tyler et al., 1993).

Generally, the infection process can be sub-divided into three phases: **pre-infection phase**, **infection phase** and **post-infection phase**.

i) The pre-infection phase

Plants have the ability to recognize invading microbes by engaging elaborate signalling systems to facilitate their communication and counter-communication with the microbial interacting partners. They have evolved unique receptor proteins on their cell surface as an adaptive mechanism to cope with the dynamic biotic environment. These receptors perceive the microbe-derived molecules and transduce the emanating signals downstream of the cell. Genetic analyses for plant microbe interactions show the phenomenon of gene-for-gene relationship where the host has genes governing resistance (R) and corresponding genes in the pathogen governing avirulence for a given host, which encode products that can be recognized by the plant hosts resulting in defense response (Flor, 1971). Pathogens also secrete effector proteins that alter the physiology of the host hence facilitating a successful colonization. These effectors can be targeted to the apoplast (the space outside plant cell membranes) or traslocated into the host cell (cytoplasmic effectors). For example, the oomycete fungi *Phytopthora infestans* secrete hydrolytic enzymes such as proteases, lipases and glycosylases that target tissue for degradation. They may also secrete enzyme inhibitors and necrotizing toxins such as the Nepl-like proteins and PcF-like small cystein-rich proteins (Haas et al., 2009). Bacteria pathogens like *Ralstonia solanacearum,* possess virulence factors such as exopolysaccharides, degrading enzymes and a type III secretion system functioning as an export system to inject virulence proteins directly into the plant cell (Chisholm et al., 2006). They also have a complex quorum sensing system to monitor their local population density through engagement of signal-response mechanisms. Bonnie (1999) reported a volatile quorum sensing signal 3-hydroxy palmitic methyl ester which the bacteria uses to sense population densities and regulate production of extracellular polysaccharides and degradative enzymes such as pectin methyl esterase, endoglucanase and polygalacturonases.

Several other substances of pathogen origin known as elicitors are recognized by the plant cells via interactions with specific receptors on the plasma membrane and stimulate different plant defense mechanisms. Elicitors can be exogenous (of pathogen origin) or endogenous (liberated from an attacked plant). They may also be specific implying that they have differential elicitor activity in various plant cultivars or non-specific elicitors lacking this differential activity. Specific elicitors are conditioned by avirulence genes in a specific pathogen which also determines the host range (Yoshikawa et al., 1993). Most fungi contain cell wall constituents acting as elicitors including the

oligosaccharide fragments such as hepta-ß-glucans, oligochitin/oligochitosan, polypeptides, glycoproteins and polyunsaturated fatty acids (Bartnicki-Garcia, 1968). The elicitor-receptor interactions generate signals that later activate nuclear genes involved in plant defense reactions including the biosynthesis of phytoalexins, programmed cell death (PCD) due to synthesis of oxidative molecular compounds e.g. reactive oxygen species (ROS), production of glycosyl hydrolyses capable of attacking surface polymers of pathogens and synthesis of proteins that inhibit degradative enzymes produced by pathogens. At the pre-infection phase, it is too early for defense gene synthesis, therefore, the host defense system is mainly characterized by formation of infection barriers such as the plant cell wall modifications by deposition of callose, hydroproline-rich glycoproteins and lignin (Hammond-Kosack and Jones, 1996).

Other pathogens such as bacteria possess the cell envelope peptidoglycan and components of the flagella. For viral pathogens, they consists of infective particles and are considered obligate intracellular parasites usually composed of positive single-stranded ribonucleic acid (RNA) and in some cases single-stranded deoxyribonucleic acid (DNA). They rely on passive means to access the cell interior e.g. through wounds, natural openings or via a vector. The viral particles have to be sensed by the host plant for an interaction to occur, but in plants, there is no evidence for recognition of viral RNA or DNA by the host receptors (Zvereva et al., 2012). Therefore, since plant viruses evolve rapidly producing new avirulence factors such as coat, movement and replication proteins resulting in resistance-breaking viral genotypes, the host plant in response has established a specialized defense mechanism known as post-transcriptional gene silencing (RNAi silencing) (Zvereva et al., 2012), able to detect and resist viral infections.

During the plant-microbe interactions, two forms of interactions may be formed, i.e. symbiotic or pathogenic interactions which initially starts with the perception of the invading microbe. During rhizobia-legume symbiosis, the host roots exudes the secondary metabolites flavanoids, that are perceived by rhizobia leading to induction of bacterial nodulation genes and eventual production of lipochitooligosaccarides (LCOs) signals referred to as Nod factors. In AMF association, the legume roots secretes sesquiterpene strigolactone that induces mycelial hyphae growth and mycorrhizal (Myc) factors that precede the association. During pathogenic interactions, microbial compounds known as general elicitors or pathogen-associated molecular patterns (PAMPS) that induce initial signalling are released (Genre, 2008; Schenkluhn et al., 2010). The establishment of these two forms of interactions is tightly regulated by the membrane-bound receptors such as the receptor-like kinases/proteins (RLKs or RLPs) which possess extracellular domains that specifically recognizes

invading microbes and an intracellular domain that mediates downstream signal transduction (Monaghan & Zipfel, 2012; Antolin-Llovera et al., 2012; Oldroyd et al., 2013). The downstream signalling pathway involves protein-protein interactions coupled with reversible protein phosphorylations that play a significant role during plant signal transduction. This entails activation of MAP-kinases cascades and several other components in the nuclear envelope such as the cation ion channel, subunits of nuclear pores and the nuclear-localized calcium/clamodulin-depedent kinase which are transported to the nucleus to phosphorylate specific transcription factors. Some members of plant receptor proteins include the Lysin Motif (LysM) family proteins which initiate formation of symbiosis either with phytobacteria or mycorrhizal fungi. The symbiosis receptor-like kinase (SYMRK) is activated at the initiation of endosymbiosis that lead to calcium oscillation patterns, while in pathogenesis specific receptor-like kinases mediate downstream activation of defense genes (Colditz and Braun, 2010). The LysM protein NFP (for nodulation factor protein) is not only involved in nodulation factor signalling, but also in pathogen recognition and resistance (Oldroyd et al., 2009). Interactions between two signal transduction pathways is shown to exist in plants, a phenomenon known as crosstalk, which is meant to furnish a rapid and efficient tuning mechanism to optimize cognitive behavioral response of plant systems (Genoud and Metraux, 2001).

ii) The infection phase

Plant microbes utilize diverse life strategies to access the plant interior. In case of motile pathogens, they must negotiate the host's surface before entering. Some pathogens of fungal origin utilize topographical cues existing on the plant surface to guide them towards a likely opening such as a stoma. Volatile compounds exuded from the pore provides a signal that triggers development of a specialized penetration structure known as appressorium that directly enter the epidermal cells as in the case of fungi. Several other microbes forming symbiotic interactions e.g. arbuscular mycorrhiza or pathogenic interactions e.g. the oomycete fungi can invaginate their feeding structures (haustoria) into the host cell plasma membrane. The pathogenic bacteria may proliferate intercellulary in the appoplast after gaining entry through the pre-existing openings on the plant surface such as stomatal pores or wounds. However, for the plant viruses, they are passively transferred into the host via vectors such as fungi or insects. During such infections, the host plant controls several processes aimed at either to enhance or hamper colonization. These include, remodeling of the cytoskeleton architecture, polarization of the plasma membrane microdomains at the penetration site and aggregation of the cell cytoplasm, secretory vesicles, endoplasmic

reticulum and vacuoles (Lu et al., 2012). In symbiotic interactions, a host-derived peri-bactereoid membrane surrounding the nodule and a peri-arbuscular membrane surrounding the arbuscules are formed during rhizobial and mycorrhizal symbiosis, respectively. These membranes form a nutrient exchange interface between the microbe and the host. In addition, the host plant controls the spatial colonization by synthesis of pre-symbiotic components, intra/intercellular reorganization, formation of plant-derived structures like the pre-penetration apparatus in case of mycorrhizal and infection threads in rhizobial symbiosis (Finlay, 2008).

Prior to successful establishment of host contact, signals from the microbial extracellular molecules are decoded by the protein sensors in the host cell, triggering specific networks and activation of initial cellular defense responses that often may be characterized by gene expression patterns and production of primary defense compounds. The hallmark of initial host defense system therefore, is to prevent the proliferation of an infection from the point of microbial invasion. The conferred immediate responses against the microbial challenge acts synergistically in rapid activation of wide repertoire of symbiotic or pathogenic specific defense responses. At this point, the host gene expression initially is supposed to be similar but later split into symbiotic specific and pathogenic specific defense response (Schenkluh et al., 2010).

A successful penetration into the host involves breaching of the host's ensued barriers, subverting or inactivating the already preformed or induced defenses as well as other specific plant-resistance mechanisms. In fungi for example, it involves adhesion to the plant surface, followed by hyphal penetration by application of pressure that is achieved from its attachment to the plant surface via proteinaceous glue. A penetration peg forms that precedes hyphal entry through the host's cuticle and the cell wall. The entry process also involves enzymatic degradation of the cuticle and the cell wall. In this case, the pathogen switches on and off a succession of genes to allow production of cutinase, cellulose, pectinase and protease that attack the cuticle, cell wall and the middle lamella. For microbes such as the necrotic bacteria, they produce also nonspecific or degradative enzymes e.g. pectinolytic enzymes that destroy the host's cell at the point of contact before colonization. They colonize the entire plant via the exylem vessels by first producing the extracellular polysaccharides to avoid recognition and cross the highly suberinized endodermal barriers, usurping the host defenses mechanisms, e.g. the tomato bacterial wilt caused by *Ralstonia solanacearum* (Kiirika et al., 2013).

During these interactions, the host plant may engage other broad spectrum of defense systems in addition to the already preformed or microbe-induced physical/chemical barriers and defense genes

that entails hypersensitive response (HR) often leading to cell necrosis or PCD localized at the infection site to restrict further microbial ingress (Andrio et al., 2013). Central to PCD is the reactive oxygen species (ROS) produced during the early infection phase and as a form of basal defense mechanism in cells. It is produced in several locations such as the plasma membrane, the mitochondria, chloroplast or peroxisomes. Superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) are the typical ROS accumulated in the host cells and can also be directly toxic to the pathogens. In addition, the host also produces several antioxidant enzymes such as peroxidase acting as interceptors to protect the cell from oxidative damage (Shapiguzov et al., 2012).

At the plasma membrane for example, H_2O_2 is produced by reduction of two-electron molecular oxygen activated by the membrane-associated NADPH oxidase homologues termed as RBOH (for respiratory burst oxidase homolgue) or spontaneously by $H_2O^{\bullet}$ or $O_2^{\bullet-}$ dismutation. Thus, during our investigations, the expression of genes such as RBOH during symbiotic interactions in *M. truncatula* was found to be low at the early phases of infection, but high after infection and again low at a later point of infection. In contrast, its expression during pathogenic interaction was high even at later points of infection (section 2.3). This gives an indication of specificity in host's gene expression in response to a given microbial invasion and a hint to a likely capability of some symbionts to suppress ROS production in cells to avoid toxicity. The lipopolysaccharide fragments (Lipid A) were documented to effectively suppress initial antioxidative burst in plants leading to subsequent suppression in expression of other defense-related genes (Tellström et al., 2007). ROS can diffuse across membranes where it may act as a second messenger during signal transduction (Borisova et al., 2012). The scavenging mechanisms of ROS in cells is controlled by the cellular antioxidative activity provided by enzymes such as superoxide dismutase (SOD), ascorbate peroxidase, glutathione peroxidase and catalase (Steffens et al., 2013). ROS scavenging leads to cell wall strengthening, which involves structural reinforcement of cell walls by cross-linking of various extracellular proteins including proline-rich glycoproteins to the polysaccharide matrix and increase in enzymatic activities such as peroxidases (Hammond-Kosack and Jones, 1996; Dj ebali et al., 2011). Whenever these primary and secondary defense barriers are usurped, a susceptible infection occurs.

iii) The post-infection phase

A successful microbial infection is achieved by establishment of an association between the microbe and the host. In the case of symbiotic interactions, a mutualistic association between the symbiont and the host is established, while in pathogenic interactions a parasitic relationship is

formed. Specific gene expression follows where the host differentiates the symbiotic specific and pathogenic specific genes. In rhizobia infection for example, genes controlling nodule organogenesis (nodulins) and those responsible for nitrogen fixation (nitrogenase) are induced. During pathogenic interactions, secondary defense pathways and associated complex molecular events are activated due to the pronounced oxidative burst and reactions from HR. This mainly consists of R proteins, the pathogenesis-related (PR) proteins such as chitinases and glucanases, endopolygalacturonases, hydroxyproline-rich glycoproteins, protease inhibitors as well as the antimicrobial compounds of secondary metabolism such as the phytoalexins and phenylpropanoids. The microbe may subvert the host cell by secreting a wide repertoire of proteins that alter the plant defense mechanisms enabling colonization of other parts of the plant (Haas et al., 2009). In response to microbial invasion, plants also activate different defense pathways depending on the pathogen encountered which comprise generation of phytohormones such as jasmonic acid and salicylic acid. During symbiosis with rhizobia bacteria, the phytohomones such as auxin and cytokinin which are considered to be important for initiation of nodule development are produced by the host. The outcome of rhizobia symbiosis is the formation of specialized organs known as nodules below the infection site, which provide oxygen-free conditions for functioning of nitrogenases responsible for nitrogen-fixation. In mycorrhizal association, both the host plant cell and the AMF undergo subcellular development where the inner cortical cells of the root branch to form the key nutrient exchange structures known as arbuscules (Finlay RD, 2008).

Phytopathogens regarded as necrotrophs pose little effects on the plant physiology as they first kill the cells before colonizing. However, for biotrophs, they invade various plant systems and subtly modify processes such as respiration, photosynthesis, translocation, transpiration, growth and development. The root infecting pathogens affect the plant's ability to absorb water by destroying the root system, producing secondary symptoms portrayed as wilting and defoliation. Several other pathogens affect the vascular system distracting water movement up the plant by blocking the xylem vessels. Eventually, the plant growth and development are affected due to changes also in source-sink pattern caused by net influx of nutrients into the infected leaves to satisfy the demands of the pathogen. Others may also interfere with the hormone balance in plants either by releasing their own hormones or triggering synthesis or degradation of phytohormones leading to malformed growth of infected parts. Finally, after pathogenic interactions, symptoms are manifested phenotypically on the host plant depending on the type of infection and the part of the plant infected which in severe cases culminates to death of the whole plant.

1.2 The model legume *Medicago truncatula*

Legumes form the third largest family of higher plants second only to Gramineae in their importance to humans and are among the 88% of the plant species that are investigated for nodule formation with the rhizobia bacteria (Doyle, 2001; Graham and Vance, 2003). Rhizobia-legume symbiosis is limited to legumes (Fabales) which belong to eurosids 1 orders (Fabideae), the nitrogen-fixing clade (Wang et al., 2006; Markmann and Parniske., 2009). Legumes include essential grain, pasture and agroforestry plant species that have proven to be essential for food and to the general ecosystem. Beans (*Phaseolus vulgaris*) and soybean (*Glycine max*), for example, are utilized as staple food in Asia, Americas and Africa and their domestication dates over 3,000 years ago (Kaplan and Lynch, 1999). Majority of legumes that are cultivated belong to *Papilionidea* subfamily consisting of the tropical or phaseolid legumes and the temperate or galegoid legumes. The phaseolid legumes include the genera *Phaseolus*, *Vigna* and *Glycine* while the galegoid legumes including the genera *Melilotus*, *Medicago*, *Pisum*, *Trifolium*, *Cicer*, *Vicia*, *Lotus* and *Lens* (Young et al., 2003).

The hallmark trait of legumes is the capacity to fix the atmospheric nitrogen via the endosymbiosis with rhizobia bacteria, considered to be due to the whole-genome duplication (WGD) approximately 58 million years ago and subsequent substantial genome rearrangements (Young et al., 2011). The occurrence of WGD resulted in additional genes including the signalling components NFP and transcription regulators ERN1 that retained their paralogues LYR1 and ERN2, respectively, expressed during mycorrhizal colonization. These genes are said to have undergone sub- or neo-functionalization and later became specialized for different roles in the initiation of nodule development. The evolutionarily ancient mutualistic symbioses with AMF can be traced back in ancient evolution over 400 million years ago, hence the RL symbiosis is much younger and its evolution is suggested to have coincided with or preceded by the bacterial endosymbiosis of Eurosid 1(Simon et al., 1993; Redecker et al., 2000; Hildebrandt et al., 2002). Moreover, genes involved in symbiosis such as the receptor kinases known as SYM genes, i.e. NORK (Nodulation Receptor Kinase) or SYMRK (Symbiosis Receptor-like Kinase) have been identified. SYM genes are essential both for bacterial and fungal symbiotic signals, which evolved in the context of arbuscular mycorrhiza symbiosis and subsequently got recruited for RL symbiosis. Considering the critical role played by legumes, there is need to understand the molecular mechanisms involved especially during legume-microbial interactions but the progress is hampered due to the fact that legumes possess large genomes. Therefore, the two model plants *M. truncatula*

(barrel medic) and *Lotus japonicus* were selected as model plants for legumes whose genome tools are established (Udvardi et al., 2005; Young and Udvardi, 2008). *M. truncatula* draft sequence of the euchromatin was published recently which captured ca. 94% of all genes (Young et al., 2011). Vast amounts of expressed sequence tags that totals to 269, 501 have been deposited in Genebank (October 08, 2013; http://www.ncbi.nlm.nih.gov/dbEST/dbEST_summary.html) as well as gene expression atlas established via an Affymetrix GeneChip that covers >50,000 gene probes (Benedito et al., 2008). Moreover, large-scaled insertional mutagenesis *Tnt1* populations and RNA interference (RNAi)-mediated gene silencing have been established in the line of reverse genetics (Limpens et al., 2004). Establishment of *M. truncatula* as a model plant for studying legume biology in 1990th therefore, bridged the gap of research in molecular biology facilitating novel investigations on Fabaceae biology, especially during symbiotic interactions in plants. This also provided an additional model plant for investigations on genome science preceded by the *Arabidopsis thaliana.*

The galegoid legume *M. truncatula* is a self-fertile plant with a diploid (2n=16) genome structure and is a close relative of *Medicago sativa* (Alfalfa), a crop that is widely cultivated. For scientific investigations on legume-specific rhizobia-legume symbiosis, *M. sativa* was and is more often considered as an object of choice since the bacteria interactions in this organism appears to be more effective. In addition, the very important phenylpropanoid flavone and phytoalexine biosynthesis pathways -the prerequisite for establishment of the legume-rhizobia symbiosis - are very well studied in *M. sativa* (Dixon et al., 2002). However, *M. sativa* has a complex autotetraploid genome and its obligate out-crossing attributes contrasts that of *M. truncatula*, hence disqualifying it for genome research in legumes (Young et al., 2011). *M. truncatula* offers other properties of a model plant for genomic analyses in legumes which include; a relatively small genome size of about 500 Mbp, half the genome size of *M. sativa* and almost ten times smaller than that of pea (ca.4340 Mbp) (Blondon et al., 1994), a short regeneration period, the ability to host majority of pathogens that infect legumes and available genetic transformation protocols. In addition, *M. truncatula* is a species of choice for studying specific pathways including the isoflavanoid pathway, a salient feature for rhizobia-legume symbiosis (Colditz and Braun, 2010). It also exhibits a high level phylogenetic relationship with other legumes crops of economic importance such as pea, beans, lentis and clover. Therefore, the high level of synteny and the biological diversity makes *M. truncatula* an agronomically relevant plant for conducting research and transferring the findings to these other crops.

1.3 Symbiotic and pathogenic interactions in legumes

The plant rhizosphere constitutes vast number of biological organisms, some of which are beneficial and others are non-beneficial. Although most of the higher plants are capable of establishing symbiotic associations with arbuscular mycorrhizal fungi, rhizobia associations culminating to nitrogen fixation is a salient feature in legumes. Mutualistic associations between legumes and rhizobia bacteria leads to formation of nodules, which are the centres for atmospheric spheric dinitrogen conversion. Legumes establish diverse interactions with microbes (Colditz and Braun, 2010) and through symbiotic interactions, they acquire macronutrients such as phosphorus and nitrogen, as well as most likely an array of micronutrients in exchange for plant-fixed carbon. (Finlay RD, 2008).

For a long time, it has been known that rhizobium and mycorrhizae symbioses in legumes share common signalling genes as well as related LCOs signalling factors. For example, the *G. intraradices* secretes symbiotic LCOs that comprise of a mixture of Nod factor-like molecules or the Myc factors. Both the Nod and Myc factors contain a tetrameric or pentameric N-acetyl glucosamine backbone with an acylated non-reducing end with C16:0 or C18:1 acyl chain or a sulfate group, indicating that both factors are related in structure. Moreover, the common signalling pathway is activated by LysM-type Nod factor receptor kinases, but the receptors MtLYK3/LjNFR1and MtNFP/LjNFR5 identified in *M. truncatula* and *L. japonicus*, are shown not to be essential for mycorrhizal symbiosis. This knowledge discloses the evolutionary origin of Nod factor receptors that specifically occurred to support the rhizobium Nod factor signalling (Streng et al., 2011). Genes that form key molecular components and essential for establishment of pre-symbiotic dialogue in plants have also been identified. Seven symbiosis (Sym) genes were identified in *L. japonicas* with four orthologues isolated from *M. truncatula*. These include; plasma membrane localized LRR-type receptor DMI2 (does not make infection) that encodes a symbiotic receptor-like kinase (SYMRK), a cation channel in the nuclear envelope DMI1 (POLLUX/CASTOR) and subunits of the nuclear pore (NUP85, NUP133), DMI3 encoding for nuclear localized complex of calcium calmodulin dependent kinase (CCaMK) and IPD3 that encodes an interacting protein of DMI3 (Markmann and Parniske, 2009; Streng et al., 2011). The CCaMK is a key component of symbiotic signal transduction playing a key role in deciphering signals downstream of the cell into calcium spiking, a process that involves fluctuations of calcium concentration in response to infection during symbiosis leading to induction of symbiotic genes. The non-beneficial plant pathogens differ genetically from saprophytes as they have genes that

control different aspects such as colonization (in the host) or parasitism (on the host), necessary for symptom expression due to general plant system dysfunction/debilitation, host-range and avirulence genes (Djordjevic et el., 1987).

i) Rhizobia symbiosis

During the establishment of legume-rhizobia symbiosis, plants initiate the dialogue by secreting exudates into the rhizosphere, the so-called flavanoids. These compounds form precursors for molecular dialogue that exist between rhizobia and the host roots. Detection of flavanoids by rhizobium triggers production of LCOs, the nodulation factors (NFs) whose perception activates the symbiosis signalling pathway. As reported for *M. truncatula*, the rhizobial morphogenic signals identified as LCOs constitute chitin made of beta, 1-4 N-acetylglucosamine backbone and are perceived by the nodulation factor receptor proteins NFP (for nodulation factor protein) and LYK (for LysM receptor kinase) in the host plant which mediates downstream cellular signalling for initiation of nodulation (Pietraszewska-Bogiel et al., 2013). Specificity in interaction between different rhizobia and host legume species is determined by the length and degree of saturation of the N-acyl group attached to the non-reducing terminal sugar of the chitooligosaccharides backbone coupled with binding of variety of substituents such as methyl, fucosyl, acetyl and sulphate groups to the acylated terminal (Roche et al., 1991). In *Lotus japonicus*, the microsymbiont *Mesorrhizobium loti* secretes a pentameric *N*-acetyleglucosamine carrying a *cis*-vaccenic acid and a carbamyl group at the non-reducing terminal residue. Receptor-like kinases with LysM RLK domains in the extracellular region function in NFs signalling thereby leading to establishment of an interaction. Two LysM RLK have been reported including the LysM 1 clade which include NF receptor 1 of *L. japonicus* and LysM receptor kinase 3 (LYK3) of *M. truncatula* and the LysM II clade which include NF receptor 5 of *M. truncatula* (Limpens et al., 2003; Radutoiu et al., 2003; Oldroyd, 2013).

Proteomic studies have identified several symbioses-induced proteins including specific nodulation-related host leghemoglobin and others such as aquaporin, an H+ -ATPase, marker proteins ENOD8, ENOD16 and nodulin-25, all isolated from the symbiosome membrane. The nodulation process involves induction of genes including the nodulation (*nod*) genes responsible for parasitism, root-hair curling, stimulation of infection-thread synthesis as well as genes that play key role in nitrogen fixation (*fix* genes) and nitrogenase (*nif* genes) both contained in the *Sym* plasmid (Catalano, 2004).

Legumes regulate the extent of nodulation using the *nodDABC* genes. This is accomplished by balancing between the stimulatory compounds mainly the hydroxylated flavones or flavanones (e.g. 7, 4, -dihydroxyflavone identified in clovers, luteolin in alfalfa, apigenin-7 -0- glycoside in peas) and inhibitory compounds such as the umbelliferone (a coumarin) and formononetin (an isoflavone) identified from peas and white clovers, and exuded from cells behind the root tip in the infection thread (Djordjevic et el., 1987). These compounds function via a competitive mechanism targeting expression of *nodD* gene, of which its pairing with the flavones/isoflovone/coumarin governs also the host-specificity during the nodulation process. The ratio of two phytohormones (auxin and cytokinin) mainly involved in initiation of nodule development is essential where the auxins acts synergistically (Gonzales-Rizzo et al., 2006). Nodule organogenesis proceeds with rhizobia invasion towards the nodule primordium and bacteria accommodation inside the nodules for nitrogen fixation which can be described as follows: After signalling process, morphological and developmental changes including calcium oscillation patterns that occurs first in the epidermal cells but later also in cortical cells, precedes bacteria colonization. Via the root hair cells, bacteria gains entry into the plant root. The root hair cells grow around the bacteria attached at the root surface, trapping the bacteria inside a root hair curl. Infection threads develop from the site of root hair curls that paves way for progressive invasion into the root tissue. At the same time, the pre-infection thread is formed that consists of relocation of the nucleus to the infection site and the alignment of the cytoskeleton and endoplasmic reticulum predicting the path of the infection thread. Underneath the infection site, nodules are formed that entraps the bacteria that are taken up into structures known as symbiosomes (Peleg-Grossman et al., 2012; Oldroyd, 2013). The IF develops towards the direction of the emerging nodules and ramifies within the nodule tissue. Bacteria are released into the membrane-bound compartments inside the cells of the nodule where they differentiate for nitrogen fixation. In some legumes such as *M. truncatula*, the nodules formed are indeterminate, which possess a persistent apical meristem while for others like *Lotus japonicus,* form determinate nodules where no apical meristem exists (Guan et al., 2013).

ii) Arbuscular mycorrhizal symbiosis

Ca. 85% of terrestrial plants establish the evolutionarily ancient mutualistic symbioses with AMF (Redecker et al., 2000; Hildebrandt et al., 2002), which can be traced back in over 400 million years ago and is said to have contributed to colonization of the terrestrial environment by land plants (Simon et al., 1993). AMF symbionts within the phylum Glomeromycota are obligate biotrophs, distributed in different natural and agricultural ecosystems (Sawers et al., 2008). In the

rhizosphere, plant releases strigolactones which are carotenoid-derived terpenoid lactones that act as signalling molecules. Perception of these molecules by the AMF activates production of Myc factors such as LCOs that are structurally related to rhizobial Nod-factors, initiating the plant- and fungal-derived exchange signals during the pre-symbiotic stage activating symbiosis signalling pathway in the root culminating to calcium oscillation patterns. This results in AMF spore germination and hyphal branching. An infection peg forms from the hypopodium that paves way for fungal growth into the root epidermal cells. A pre-penetration apparatus is formed in the cell from the clustering of endoplasmic reticulum and cytoskeleton which predetermines the growth path of the fungal hyphae within the cells (Genre et al., 2008; Parniske et al., 2008). Through a coordinated subcellular development of the host plant cell and the AMF, the root inner cortical cells branch to form arbuscules, the key nutrient exchange structures.

iii) Pathogenic infection:

Plants face potential pathogenic microoorganisms which may include fungi, bacteria, virus or nematodes. To be pathogenic, these organisms must access the plant interior and overcome or subvert obstacles such as the cell wall and a rigid cellulose armament. Further on, they encounter also the plasma membrane which has extracellular receptors able to recognize a broad swath of microbial signatures, the so-called PAMPS and trigger innate host defense response (Chisholm et al 2006). PAMPs include fungal features such as chitin, oomycete cell wall beta-glucans, common bacteria cell envelope component peptidoglycan, breakdown of plant cell wall products secreted molecules and peptides unique to individual pathogens as well as components of the flagella elongation factors (Peleg-Grossman et al., 2012). Plants may activate systematic acquired resistance that provides them with a lasting defense response transmitted to all parts of the plant upon onset of an infection (Shah et al., 2009. The defense system can also be triggered exogenously using elicitors to mimic the signalling molecules in a pathogen as reported for tomato (*Solanum lycopersicum*) against bacterial wilt caused by *Ralstonia solanacearum* (Kiirika et al., 2013).

The model legume *M. truncatula*, hosts both fungi (Ameline-Torregrossa et al., 2008); bacteria (Turner et al., 2009) and oomycetes pathogens such as the root rot oomycete of the genera *Pythium, Phytopthora,* and *Aphanomyces* (Colditz et al., 2005; Colditz and Braun et al., 2010). The oomycetes are a large class of eukaryotes that is composed of 600 to 1500 species and is phylogenetically different from the “true” fungi but this class constitutes the most numerous, most important and earliest known water moulds. Studies have been carried out to understand the pathosystem existing between *M. truncatula* and the biotrophic oomycete pathogen *Aphanomyces*

euteiches in legumes (Gaulin et al., 2007). Two *M. truncatula* accession lines A17 and F83005.5 have been shown to possess contrasting responses to *A. euteiches* infection where the former is partially resistant and the latter susceptible (Colditz et al., 2005).

A. euteiches is a devastating pathogen in legumes especially peas (*Pisum sativum*) and is regarded as one major yield-reducing factor in legume crop production (Colditz et al., 2004; Gaulin et al., 2007). It produces motile spores known as zoospores that function as infection carriers. During the infection process, the pathogen projects its specialized hyphae and houstoria into the host cells. The plant performs cellular reorganization including biogenesis of novel cell membrane and an extrahaustorial membrane that encysts the pathogen haustorium eliminating a direct link between the host cell and the pathogen. The pathogen subverts the host cell by secreting a wide repertoire of effector proteins such as proteases, lipases and glycosylases that modulate the host innate defense, hence facilitating a successful parasitic infection and colonization of the plant tissue. After an infection, symptoms of *A. euteiches* appear on the roots since it's a root-infecting pathogen, progressing into the stem tissues. The infected root tissue appears yellow and at a later stage becomes honey-brown with the hypocotyl darkening at the soil line. Eventually, the root mass reduces followed by necrosis and death of the whole plant. Cataloguing of putative genes controlling the pathogenicity of *A. euteiches* has been enabled by availability of genome sequences for some of the most important plant pathogenic oomycetes species such as *Phytopthora* (Tyler et al., 2006), *Pythium* (Lévesque et al., 2010), *Hyaloperonospora* (Baxter et a., 2010) and *Albugo* (Kemen et a., 2011). For the oomycete pathogen *A. euteiches*, the analyses revealed two cDNA libraries from mycelia isolates that were either from the model legume *M. truncatula* or synthetic medium comprised of more than 18,000 ESTs assembled to nearly 8,000 unigenes accessible in the 'AphanoDB public database' (Madoui et al., 2008). The analyses of *Phytopthora* spp. (*P. infestans*) genome revealed an extensive expansion when compared to the genomes of the other two spp. *P. sojae* and *P. ramorum* which is as a result of a repetitive proliferation of DNA regions encoding families of manifold secreted disease effector proteins.

1.4 Objectives of the thesis

The overall objective of this thesis is to characterize the model legume *Medicago truncatula* during the early phases of microbial interactions via molecular and proteomic approaches. This entails gene and protein expression analyses combined with morphological and physiological observations of plants during symbiotic interactions with nitrogen fixing rhizobia bacteria, *Sinorrhizobium meliloti* and arbuscular mycorrhizal fungi *Glomus intraradices* as well as oomycete pathogen *Aphanomyces euteiches*. It is conducted on the background of RNA interference gene silencing of MtROP9 to characterize the functional role of small GTPases of plants in the context of signal transduction network during microbial infection. This study provides new insights into the understanding of molecular mechanisms underlying symbiotic and pathogenic interactions in legumes.

In the following chapter, three investigations that correspond to the objectives are presented:

In **section 2.1**, the key role of Rac1 GTPase MtROP9 in ROS-mediated early infection signalling is presented. This study reports that silencing of the Rac1 GTPase MtROP9 in *M. truncatula* stimulates early mycorrhizal and oomycete root colonizations but negatively affects rhizobial infection. Data on genetic and proteomic characterization as well as morphological and physiological analyses are displayed.

Establishment of plant-microbe interactions requires energy metabolism mainly taking place in the mitochondria. In **section 2.2**, the mitochondrial complexome of *M. truncatula* is presented via the novel GelMap annotation tool. Several of the complexes identified in this study are hereby described for the first time in *M. truncatula* based on comparative analysis with the already well-studied model plant *Arabidopsis thaliana*.

In **section 2.3**, the infection proteome of the MtROP9-interfered *M. truncatula* roots and the significant changes during the ROS defense signalling is presented. The data on proteome response of *M. truncatula* roots to symbiotic and pathogenic infection under conditions of suppressed potential for ROS synthesis as a common and early defense mechanism is displayed. A new approach of presenting large proteome datasets involving the Heatmap integration to the GelMap software tool is hereby described for the first time. Data presented in **sections 2.2** and **2.3** is based on the newly developed legume-specific database (LegProt) that enabled high protein identification rates. The two studies have established a good foundation and a perspective for future research on ROS defense signalling to identify and characterize the infection signalling networks in the plant mitochondria.

CHAPTER 2

Publications and manuscripts

This thesis comprises of three manuscripts. The first two are already published and the third is ready for submission to the international journal "Frontiers in Plant Science". Because all these manuscripts are multi-author manuscripts, individual author's contribution is hereby outlined:

The first manuscript 'Silencing of the Rac1 GTPase MtROP9 in *Medicago truncatula* stimulates early mycorrhizal and oomycete root colonizations but negatively affects rhizobial infection' was published in scientific journal 'Plant Physiology (159: 1-16) in 2012 by Kiirika LM, Bergmann HF, Schikowsky C, Wimmer D, Korte J, Schmitz U, Niehaus K, and Colditz F. All the authors were affiliated with Leibniz Univesität Hannover, Institute of Plant Genetics, Department of Molecular Biology, except the 2nd, 4th and the 7th authors who were affiliated with University of Bielefeld, Department of Proteome and Metabolome Research when work on this manuscript was initiated. Colditz F and Niehaus K provided the first idea and the scientific framework for conducting research with the Rac1/ROP GTPases in *M. truncatula* roots during microbial infections. I performed the RNAi-mediated gene silencing, phenotypic evaluation, infection assays, microscopic characterization of infected roots, gene expression analyses using RT-PCR and proteomic analyses. RNAi vector construct was prepared by Bergmann HF, Colditz F while parts of Semiquantitative RT-PCR were done by Korte J. Preparation of samples for ROS measurements were done by me, while Wimmer D took the luminenscence readings. Figures and statistical analysis were carried out by Colditz F, with statistical software and technical support offered by the Institute of Biostatistics, Leibniz Universität Hannover. The manuscript was written by Colditz F, with corrections done by Schmitz U. Niehaus K did the proof-reading of the manuscript. **The second manuscript** 'The mitochondrial complexome of *Medicago truncatula*' was published in the scientific journal 'Frontier in Plant Science (DOI: 10.3389/fpls.2013.00084) in 2013 by Kiirika LM, Behrens C, Braun HP, Colditz F. I was responsible for all data acquisition, preparation of figures and tables for creating the *Medicago truncatula* GelMap under the guidance of Colditz F, while Behrens C supported with Mass Spectrometry analyses. Colditz F and Braun HP wrote the manuscript. **The third manuscript** 'Proteomic profiling of transgenic *Medicago truncatula* roots defective in ROS signalling after early symbiotic and pathogenic microbial infections' is ready to submit for publication in the scientific journal 'Frontiers in Plant Science' 2013 by Kiirika LM, Schmitz U and Colditz F. All experiments and figures in this manuscript were done by me, with the guidance of

Colditz F. I prepared all the tables and figures for creating the Heatmap-Gelmap. The manuscript was written by me and subsequent corrections done by Colditz F.

2.1 Silencing of the Rac1 GTPase MtROP9 in *Medicago truncatula* stimulates early mycorrhizal and oomycete root colonizations but negatively affects rhizobial infection.

Kiirika, LM., Bergmann, HF., Schikowsky, C., Wimmer, D., Korte, J., Schmitz, U., Niehaus, K. and Colditz, F.
Plant Physiology 2012, 159: 501-516.

Link:
http://www.plantphysiol.org/content/159/1/501.abstract?sid=cd7b123e-cf1d-45b4-8829-a68069effe8b

2.2 The mitochondrial complexome of *Medicago truncatula*.

Kiirika, LM., Behrens, C., Braun, HP. and Colditz, F.
Frontier in Plant Science 2013, DOI: 10.3389/fpls.2013.00084.

Link:
http://www.ncbi.nlm.nih.gov/pubmed/23596449

2.3 Proteomic profiling of transgenic *Medicago truncatula* roots defective in ROS signalling after early symbiotic and pathogenic microbial infections.

Kiirika, LM., Schmitz, U. and Colditz, F.
Frontiers in Plant Science 2014, (*In preparation*).

Proteomic profiling of transgenic *Medicago truncatula* roots defective in ROS signalling after early symbiotic and pathogenic microbial infections

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Running title:

Medicago truncatula MtROP9i infection proteome

Keywords: *Medicago truncatula,* pathogenic interactions, symbiotic interactions

Abstract

Plant-specific small GTPases RAC/ROP (Rho proteins of plants) function as molecular switches within elementary signal transduction pathways, regulating generation of ROS (reactive oxygen species) via the activation of NADPH oxidase homologue of plants termed RBOH (for respiratory burst oxidase homologue). The level of ROS in plant cells is tightly controlled by specific protein networks to balance between toxicity and as essential molecules during microbial infection. Previously, we reported that silencing of MtROP9 in *Medicago truncatula* roots led to reduced ROS production and suppressed induction of ROS-related enzymes such as RBOH and catalase in MtROP9i transgenic roots infected with oomycete pathogen *Aphanomyces euteiches*. Furthermore, fungal infections with *A. euteiches* and arbuscular mycorrhizal fungi *Glomus intraradices* were promoted while infection with rhizobia bacteria *Sinorhizobium meliloti* was impaired. Therefore, in the current

study, we investigated the temporal proteome response of *M. truncatula* MtROP9i transgenic roots during symbiotic and pathogenic interactions under conditions of deprived potential for the plant to synthesize ROS as a common and early defense mechanism. Using the Delta2D software, proteome maps of proteins from infected roots were analyzed to decipher protein spots of different abundance, which were then systematically subjected to mass spectrometry (MS) for analysis. Protein annotation using legume-specific databases (LegProt) improved the identification rates giving a huge proteome dataset, hereby presented via heatmap-GelMap.

Out of the three infections with *S. meliloti*, *G. intraradices* and *A. euteiches,* 733 spots were found to be different in abundance. 213 spots comprising 984 proteins (607 unique proteins) were identified after *S. meliloti* infection, 230 spots comprising 796 proteins (580 unique proteins) after infection with *G. intraradices* and 290 spots comprising 1240 proteins (828 unique proteins) after infection with the *A. euteiches*. Over all, the number of induced proteins considering both the MtROP9i and Mtvector were high in pathogenic infections than with the symbiotic infections. Additionally, the number of induced proteins in MtROP9i was low as compared with Mtvector roots. Qualitative analysis of induced proteins for the three infections showed that enzymes linked to ROS production and scavenging as well as hypersensitive reaction were highly induced in Mtvector as compared with the MtROP9i, where majority were involved in alternative defense pathways such as cell wall degradation and protein degradation. The RBOH1 (induced 55 fold at 5h), peroxidase (84 fold at 5h), superoxide dismutase (46 fold at 5h), glutathione S-transferase (23 fold at 5h) and thioredoxin h1 (22 fold at 5h) were among the highly induced proteins in Mtvector roots, but with low inductions in MtROP9i roots. Cell wall degrading enzymes such as endochitinases (33 fold at 5h) and polygalacturonase inhibitor (22 fold at 24h) as well as protein degrading enzymes such as SGT1 homologue (77 fold at 3h) and PR10-1 (52 fold at 3h) were among the highly induced proteins in MtROP9i roots. Key proteome changes during symbiotic and pathogenic interactions under conditions of hampered ROS synthesis were identified. This study shows the dual functional role of ROS in defense signalling and symbiosis as well as other alternative cellular response patterns activated during microbial infection when the NADPH-mediated ROS production system is interfered.

1. Introduction

RAC/ROP (Rho of plants) are plant-specific small GTPases that function as simple binary molecular switches within elementary signal transduction pathways by cycling between GTP-bound on modes and GDP-bound off modes. In the GTP-bound forms, they interact with specific effectors downstream of the cell, mediating a wide repertoire of molecular stimuli that provoke cellular responses (Poraty-Gavra et al., 2013). ROPs integrates many upstream signals via the guanine nucleotide exchange factors (GEFs), guanine nucleotide dissociation inhibitors (GDIs) and GTPase-activating proteins (GAPs), regulating downstream effectors such as Rop-interactive CRIB motif-containing proteins (RICs) and interactor of constitutively active ROPs (ICRs) (Nagawa et al., 2012). Small GTPases are well studied in mammals and yeast cells and have been grouped into various subfamilies depending on their functional properties (Schiene et al., 2000). ROPs are known to function in different developmental processes including polarized cell growth, pollen tube and root hair development, hormonal signalling as well as cell morphogenesis (Yang and Fu, 2007; Liu et al., 2009). They are also implicated in regulating several cellular processes including vesicle trafficking, cytoskeleton organization and dynamics, auxin transport and response to pathogens (Nibau et al., 2006; Ylovsky et al., 2008; Lorek et al., 2010; Wu et al., 2011; Poraty-Gavra, 2013). The ICRs have also been shown to regulate polarized secretion and polar transport of auxin during auxin-regulated development (Hazak and Yalovsky, 2010). ROP proteins form key regulatory elements for reactive oxygen species (ROS) generation in plant cells especially at the plasma membrane by activating the NADH oxidases termed as RBOH (for respiratory burst oxidase homologue). In *Medicago truncatula*, 16 putative ROPs were suggested from assembled EST sequences (Yuksel and Memon, 2008) but only seven ROPs have been confirmed (Liu et al., 2012) with the rest either being the artifacts or redundant. In *Arabidopsis thaliana,* 11 ROPs are identified (Winge., et al., 1997).

During the initial microbial invasion, the host plant confers a general defense reaction characterized by rapid activation of wide repertoire of symbiotic or pathogenic defense cellular responses. More often, the reactive oxygen species (ROS) especially H_2O_2 forms the hallmark of these very early host defense systems that causes a hypersensitive reaction culminating to host cell death at the site of infection for the pathogen and in the case of a symbiont, contributes to its establishment (Puppo et al., 2013). ROS is also said to diffuse across the cell membranes via the aquaporins and function as second messenger during signal transduction pathway hence acting as elementary signal molecules for activation of plant defense responses (Borisova et al., 2012). The superoxide and hydrogen peroxide are the typical ROS

accumulated in host cells. The delicate balance between production and scavenging activity allows the duality in function of ROS to exist in plant system orchestrated by a large network of enzymes and antioxidative compounds. The scavenging activity of ROS in the mitochondria is controlled by the alternative oxidase (AOX), non-proton-pumping, alternative type II and the Ca^{2+}-dependent NADPH dehydrogenase (Steffens et al., 2013). The antioxidative activity in the cell is provided by molecules such as glutathione, tocopherols, tannins, phenolic compounds and ROS scavenging enzymes such as superoxide dismutase (SOD), ascorbate peroxidase, glutathione peroxidase, catalase as well as other non-enzymatic proteins such as metallothioneins and thioredoxin that lead to ROS homeostasis (Steffens et al., 2013). The ROS scavenging mechanisms lead to cell wall structural reinforcement by cross-linking of various extracellular proteins including proline-rich glycoproteins to the polysaccharide matrix (Djebali et al., 2011). In rice (*Oryza sativa*), the GTPase OsRac1 was shown to positively regulate disease resistance by stimulating the NADPH-mediated ROS production via direct binding to the catalytic subunit of NADPH oxidase N-terminal extension specific for RBOH proteins (Kawasaki et al., 2006; Jones et al., 2007; Nakashima et al., 2008). *In vivo* fluorescence resonance energy transfer (FRET) analysis showed that the Ca^{2+} concentration in the cytosol may regulate the RBOH-Rac interaction, hence modulating the activity of NADPH oxidase in ROS production (Wong et al., 2007).

A legume (Fabaceae) interacts with soil-borne microbes (Colditz and Braun, 2010) and are unique in establishing symbiosis with rhizobia bacteria, which ultimately leads to nitrogen fixation in the formed structures known as nodules. Through their association also with arbuscular mycorrhizal fungi, legumes benefits by acquiring macronutrients including phosphorus and nitrogen, as well as most likely an array of micronutrients in exchange for up to 20% of the plant-fixed carbon (Finlay RD, 2008). ROPs are shown to play a key role during rhizobia infection in the process of nodule development and also during the establishment of mycorrhizal association (Berken, 2006). Expression of MtROP3, MtROP5 and MtROP6 in *M. truncatula* increased after rhizobia inoculation as reported by Liu et al. (2010), while in *L. japonicus*, the LjROP6 was shown to act as a positive regulator of infection thread formation during rhizobia infection (Ke et al., 2012).

The current work aimed at evaluating the temporal root proteome of *M. truncatula* MtROP9i, defective of ROS synthesis in order to address the question of whether the ROP-like GTPases affects the proteome response during symbiosis of rhizobial bacteria, arbuscular mycorrhiza as well as the pathogenesis with the oomycete *A. euteiches* at the very early points of infection. The *Arabidopsis*

thaliana ROP1 and ROP6 were shown to promote symbiotic fungal associations with the root-colonizing endophytic fungus *Piriformospora indica* by forming F-actin bundles in the roots required for fungal growth (Venus et al., 2013). Previously, we reported that silencing of MtROP9 affects rhizobial infection but positively regulates root colonization by arbuscular mycorrizal fungi *G. intraradices* and oomycete pathogen *A. euteiches* (Kiirika et al., 2012). The infection process in MtROP9i transgenic roots was characterized by clear reduction of ROS accumulation in the cells, antioxidative cellular components marked by suppression in relative expression of ROS-related enzymes such as RBOH and catalase. Both symbiotic and pathogenic interactions are known to induce oxidative burst coupled with induction of defense-related products such as PR proteins, where the difference in the two forms of interactions is suggested to be of quantitative in nature especially with regard to ROS production. In addition, the generation of ROS suppresses the expression of PR genes (Peleg-Grossman et al., 2012). During symbiosis, ROS induction is shown to be exclusively localized in the cytosol while in nonsymbiotic interaction, the ROS induction is dispersed in the cytosol (Ashtamker et al., 2007). In *M. truncatula*, the MtSpk1 gene encoding a putative protein kinase was induced by exogenous application of H_2O_2 as well as nodulation factor indicating the functional role of ROS in regulating genes directly linked to rhizobia symbiosis (Andrio et al., 2013). Transient decrease in MtRBOHs gene expression was reported to lead to decrease in ROS efflux observed 1 h after *M. truncatula* roots treated with NF (Lohar et a., 2007). We also reported an impaired rhizobial infection in *M. truncatula* plants deficient of signalling protein MtROP9, known to regulate NADPH-mediated ROS production. The MtROP9i roots showed extremely swollen noninfected root hairs and impaired nodulation depicting intrinsic role of ROPs protein in ROS-mediated infection signalling and especially in establishment of rhizobia symbiosis (Kiirika et al., 2012).

In this study, we have utilized the previously investigated sequence of MsRac1 sequence from *Medicago sativa* for RNA interference (RNAi)-mediated gene silencing in the model legume *M. truncatula* where we identified a *M. truncatula* sequence ortholog annotated as MtROP9 (TC173331; Dana-Farber Cancer Institute *M. truncatula* Gene Index [MtGI]; Quackenbush et al., 2001). A gene-specific region of MtROP9 was selected for RNAi gene knockdown with *Agrobacterium rhizogenes* used as a vector. Evaluation of MtROP9i root proteome maps via 2D IEF SDS-PAGE and MS after symbiotic and pathogenic interactions at the very early timepoints of infections revealed changes in protein profiles as clearly visualized using the heatmap-GelMap tool.

2. Results and discussion

2.1 Experimental setup

The aim of this study was to evaluate the temporal proteome response of *M. truncatula* roots to symbiotic and pathogenic infection under conditions of significantly suppressed potential for the legume host to synthesize ROS as a common and early defense mechanism. We therefore generated transgenic *M. truncatula* (Jemalong A17) root populations expressing an RNAi hairpin construct for the RAC-type GTPase MtROP9 (TC173331; MtGI at Dana-Farber Cancer Institute; Quackenbush et al., 2001) as described and characterized by us previously (Kiirika et al., 2012). The transgenic roots termed MtROP9i revealed significant reduced levels of ROS production as compared to transgenic empty vector control (Mtvector) roots (Kiirika et al., 2012). As a consequence, MtROP9i roots exhibited clearly altered infection levels when inoculated with symbiotic and pathogenic microbes (Kiirika et al., 2012).

Twelve individual populations of MtROP9i were constructed independently via *A. rhizogenes*-mediated root transformation, each representing at least 200 stably growing composite plants with transgenic roots. For our infection assays and proteomic analyses, only MtROP9i and Mtvector roots comprising on average 60% and more of transformed (transformation marker/*Ds*RED-positive) roots were considered. For validation of the effective knockdown in MtROP9i transgenic roots, MtROP9 transcript abundance was determined by reverse transcription (RT)-quantitative PCR as previously described (Kiirika et al., 2012). The relative expression of MtROP9 was drastically reduced about >90 % in these root populations and did not increase after microbial infection when compared with Mtvector roots (data not shown). Importantly, MtROP9i transgenic roots of all considered individual population revealed no significant ROS production nor its accumulation after infections with the chosen compatible root microbes as confirmed via *in planta* luminometric ROS assays while Mtvector roots did (data not shown). Inoculation assays were conducted using Rhizobial bacteria *S. meliloti* as well as arbuscular mycorrhizal fungus *G. intraradices* for symbiotic interactions, and the legume root pathogen *A. euteiches* (oomycota). Proteins from four independent isolations for four timepoints of harvest (1, 3, 5 and 24hpi) were separated using two-dimensional (2-D) IEF/SDS PAGE and three of the proteome maps were selected for Delta 2D analysis in order to decipher protein spots with significantly differential abundance and later, a representative gel selected for MS analyses (fig. 1).

2.2 Proteomic profiling of MtROP9i and Mtvector transgenic roots during symbiotic and pathogenic interactions

To identify changes in the proteomes of *M. truncatula* MtROP9i transgenic roots infected with *S. meliloti*, *G. intraradices* and *A. euteiches*, proteome maps of four early infection points (1 h, 3 h, 5 h and 24 h) were prepared. Three Coomassie-stained gels representing three biological replicates and showing similar spot pattern were prepared per timepoint and evaluated by Delta2D v4.4 software (Decodon GmbH). Using the student's t-test (confidence interval $\geq$95%), the significant changes in spot pattern depicted as differences in spot abundance (>1.5 fold) for different infections were determined based on the spot volume and selected for further analysis. The spots were excised from the Coomassie-stained gels using a GelPal Protein Excision manual spot picker (Genetix, Great Britain), then in-gel digested with Trypsin as described by Klodmann et al. (2010) and analyzed via nLC ESI-MS. Protein identification was carried out based on the *Medicago*-specific protein databases from the LegProt db (Lei et al., 2011), which allowed high rates of protein identification. The protein search tool, ProteinsScape 2.0 (Bruker Daltonics) and MASCOT search engine were used for querying the three Medicago-specific databases [*Mt3.5 ProteinSeq, NCBI Medicago truncatula protein* and *Mtf*(*asta*)^2] (Lei et al., 2011) as well as *Swissprot*.

Out of the three infection assays conducted, 733 spots were identified to be of different in abundance for the four considered timepoints. 213 spots from *S. meliloti* infection were analyzed revealing a total of 984 proteins of different abundance that comprised 607 unique proteins. 385 and 568 proteins were identified in MtROP9i and Mtvector, respectively. 230 spots of different abundance were found after *G. intraradices* infection, where 796 proteins were identified revealing a total of 580 unique proteins. 311 and 485 proteins were induced in MtROP9i and Mtvector, respectively. Infection with *A. euteiches* revealed 290 spots of different abundance giving a total of 1240 proteins whereof 828 were unique proteins. 456 and 784 proteins were induced in MtROP9i and Mtvector, respectively.

Overall, the total number of induced proteins identified in MtROP9i transgenic roots after symbiotic and pathogenic infections was lower compared to Mtvector which can be attributed to knock down of the signalling protein MtROP9. However, the highest individual protein induction levels were found in Mtvector roots infected with *S. meliloti* as compared to fungal infections with *A. euteiches* and *G. intraradices* (fig. 1 and supplementary table 2). Considering the induction pattern along a timeline for the three infections, the induction level was high at the early points of infection in Mtvector with majority of proteins reaching maximum induction even at 3 hpi as compared to MtROP9i. Low protein

induction at the early infection timepoints in MtROP9i roots indicates the absence of the first line of defense comprised of ROS defense signalling. The early cellular defense reactions were affected after silencing of the signalling protein MtROP9, where also majority of other enzymes not directly related to ROS such as those involved in cell wall, protein degradation as well as PR proteins were highly induced especially during the advanced stages of infection.

All identified proteins were further classified into their physiological functions and ordered based on the most predominant physiological categories including the defense response, stress response, signal transduction and secondary metabolite biosynthesis. Proteins with the highest differential abundance in each infection were filtered and visualized on a two-coloured gel image channel (fig.1). Evaluation of proteins in these categories revealed suppressed induction of ROS-related enzymes in MtROP9i transgenic roots as compared to Mtvector roots. In this category, 18 and 43 proteins were differentially induced in MtROP9i and Mtvector, respectively, after *S. meliloti* infection, while 29 and 42 proteins were induced in MtROP9i and Mtvector, respectively in *G. intraradices* infection. In *A. euteiches* infection, 31 and 69 proteins were induced in MtROP9i and Mtvector, respectively. These proteins are intuitively linked to ROS production, hypersensitive response reaction as well as enzymes responsible for ROS scavenging. They include RBOH1 (55 fold at 5h) and peroxidase (84 fold at 5h) induced after *S. meliloti* infection in Mtvector as compared to MtROP9i roots. SOD [Cu-Zn] (46 fold at 5h), RBOH1(21 fold at 5h), peroxidase 1 (25 fold at 3h) and peroxidase pxdc (7 fold at 5h) were induced after *A. euteiches* infection as well as cationic peroxidase 1 (43 fold at 3h), glutathione peroxidase (23 fold at 5h), peroxidase 2 (23 fold at 3h) and thioredoxin h1 (22 fold at 5h) after *G. intraradices* infection in Mtvector compared to MtROP9i transgenic roots.

Proteins that were found be highest in abundance after *S. meliloti* infection include SGT1 homolog (77 fold at 3h), prohibitin (73 fold at 24h), germin-like protein subfamily 2 member 3 (43-fold at 24h) and isopentenyl pyrophosphate isomerase (45 fold at 24h) in MtROP9i roots, while pectinesterase (62 fold at 5h), peroxidase (84 fold at 5h), nodule-specific cysteine-rich peptide 96 (78 fold at 3h) and dihydroflavanol-4-reductase 1 (34 fold at 3h) were found in Mtvector roots (supplementary table 2). In *G. intraradices* infections, PR10-1 (6 fold at 5h), inosito-3-phosphate synthase (23 fold at 5h) and NAD(P)H-dependent 6`-deoxychalcone synthase (24 fold at 24h) were identified in MtROP9i roots while 14-3-3-like protein (34 fold at 3h), cationic peroxidase 1 (43 fold at 3h) and progesterone 5-beta-reductase (16 fold at 3h) in Mtvector roots. In *A. euteiches* infection, protein P21 kinase inhibitor (50 fold at 3h), isoflavonoid xylocosyltransferase (4 fold at 5h), and ricadhesin receptor, germin-like

protein (9 fold at 3h) were identified in MtROP9i while PR10-1 protein (50 fold 3h), peroxidase 1(25 fold at 3h), and hydroxycinnamoyl-CoA quinate hydroxy-cinnamoyltransferase (21 fold at 5h) in Mtvector roots (figure 1and supplemtary table 2).

2.3 General evaluation of protein induction pattern via Heatmap-GelMap

To explicitly present the voluminous protein dataset comprising of all significantly induced proteins from the three infections, i.e. *S. meliloti*, *G. intraradices* and *A. euteiches*, a technique of applying the novel software GelMap (http://www.gelmap.de) which is utilized for protein annotation was used (Kloddman et al., 2011). Heatmap comprising the three infections was integrated to GelMap module allowing clear visualization of induction patterns of all identified proteins found to be different in abundance (>1.5). This technique utilizes the inbuilt function filters in the software for any user-defined cluster of choice, based on specific protein physiological function. The Heatmap was created by clustering together all proteins of similar physiological functions both for major and sub-categories. The total inductions per sub-category presented as individual values were automatically filtered and displayed on a matrix. The heatmap was generated by a colour-coding system which ranged from dark-red to light-red corresponding to high inductions and low inductions, respectively. Proteins with the highest levels of induction either in MtROP9i or Mtvector were selected or ranked first as the most predominant physiological categories

- The total number of significantly induced proteins identified in MtROP9i transgenic roots after symbiotic and pathogenic infections were lower as compared to Mtvector indicating that the infection proteome network was affected after silencing the signalling protein MtROP9. The individual protein inductions following rhizobia infection with *S. meliloti* was the highest compared to fungal infections with pathogenic oomycete *A. euteiches* and symbiotic interactions with *G. intraradices*. Typically, this would be unexpected since *S. meliloti* is not a pathogen and hence would not pose a severe challenge on the plant defense cues. The high inductions in rhizobia infections could be attributed to the high similarity it shares with the pathogenic bacteria as shown via phylogenetic analyses based on their sequences (Willems and Collins, 1993), implying that the rhizobia bacteria may have been a pathogen but shifted into a symbiont in the course of evolution. In addition, several other protein partners may have been constitutively induced to promote formation of rhizobia symbiosis such as the nodule-specific cysteine-rich peptide 96, dihydroflavanol-4-reductase 1. However, the total number of individual proteins induced in *A. euteiches* infections was higher which is true for pathogenic infection, where it can be surmised that the host in addition to induction of the early ROS defense signalling enzymes, engages other

alternative pathways particularly at the advanced stages of infection process including induction of cell wall degrading enzymes such as polygalacturonase inhibitor, pectinesterase and SGT1protein as well as PR proteins.

- Induction of proteins involved in ROS production and detoxification was significantly reduced in MtROP9i roots as compared to Mtvector roots. RBOH 1 and RBOH B were highly induced at the early points of *S. meliloti* infection particularly in Mtvector but clearly reduced in MtROP9i. In addition, their induction levels considering *G. intraradices* and *A. euteiches* infections were low in Mtvector. SOD [Cu-Zn] was highly induced after *S. meliloti* infection, but its induction in MtROP9i roots was noted only for the three timepoints 3, 5 and 24 hpi. The decline in induction of proteins responsible for ROS production and the concomitant increase of detoxifying enzymes is indicative of the essential role of ROS during root and nodule development (Puppo et al., 2013). This is also evidenced by reduced induction of these enzymes in *A. euteiches* and *G. intraradices* infections. Accumulation of ROS during rhizobium-legume interaction could have multiple functions, one as part of a typical defense response to limit bacteria entry, second, as compounds needed for progression of infection thread or as signals for symbiotic genes synthesis (Soto et al., 2011).
- Proteins involved in antioxidative and peroxidase activity such as the thioredoxins were induced mainly at 3 and 5hpi, especially after *A. euteiches* infection. The cytosolic form of thioredoxin h functions in response to oxidative stress which may have occurred at the early points of microbial infection, mainly pronounced in the Mtvector roots. Thioredoxin also accumulates in self-incompatibility reactions, seed germination and early seedling development (Chi et al., 2013).
- Paltry induction of ROS-related enzymes in MtROP9i transgenic roots after infection could not be ruled out especially after *S. meliloti* infection. This could be attributed to remnant fragments of MtROP9 protein that may been left out after its knockdown or even the presence of other related GTP binding protein partners that as a consequence, could potentially contribute to ROS synthesis at the cellular plasma membrane. Such proteins which belong to the category GTP binding/GTPase activity include the nuclear protein Ran/TC4, dynamin-related protein and guanine nucleotide-binding protein.
- Protein specific for rhizobia infection, the nodule-specific cysteine-rich peptide responsible for early nodulation signalling was highly induced at 3hpi with *S. meliloti* in Mtvector roots. The predominant induction of this protein very well indicates the success of rhizobia infection in Mtvector roots, not present in MtROP9i roots suggesting an evidence for hampered progress in rhizobia symbiosis as previously reported (Kiirika et al., 2012)

- Major latex protein (MLP) and ABA-response protein (ABR17) which are in the PR proteins category were predominantly induced after *A. euteiches* and *G. intraradices* infections at 5 hpi in Mtvector compared to MtROP9i. These proteins are induced when the intracellular ABA concentration increases in the cell during the course of infection. PR proteins comprising class 10, 1A, 5-1 and 1 as well as osmotin/thaumatin-like proteins were induced particularly in Mtvector roots infected with *S. meliloti*. Induction of PR proteins was previously reported both at the transcript and protein level (Colditz et al., 2004, 2005) forming the major components of molecular host defense response on *M. truncatula* after *A. euteiches* infection both at the early and later infection stages. The induction of PR proteins, in particular, was detected distinctively at advanced stages of infection (5 and 24 h) with predominantly high inductions in Mtvector as compared to MtROP9i roots. So far, reports indicate that 14 classes of PR protein (PR1-PR14) are known in plants (Spoel et al., 2012). Early reports on engineering efforts in plants for diseases resistance through over expression showed that PR proteins are effective when coordinately expressed in plants than when induced individually (Loon et al., 1999). Previously, it was reported that both PR protein classes were conjointly modified in expression following *A. euteiches* infections in *M. truncatula* (Trapphoff et al., 2009; Colditz et al., 2007).
- Several other proteins differentially induced were classified in the category of defense response, proteins involved in protein degradation, proteolysis and proteolysis inhibitor, induced only after *S. meliloti* and *A. euteiches.* In the category of secondary metabolite and biosynthesis proteins responsible for phenylpropanoid biosynthesis (e.g. hydroxycinnamoyl-CoA quinate hydroxycinnamoyl-transferase, phytoalexin biosynthesis (e.g. chalcone-flavonone isomerase 1, isoflavonoid xylocosyltransferase and NAD(P)H-dependent 6'-deoxychalcone synthase) and flavonoid biosynthesis (e.g. dihydroflavanol-4-reductase 1 and CXE carboxylesterase) were induced. Structural protein (e.g. MFP1 attachment factor), hormone metabolism (e.g. abscisic acid receptor PYR1 and cytokinin-O-glucosyltransferase), cell division and development (e.g. actin, profiling and cyclin) and lipid/fatty acid metabolism (e.g. phospholipase D, fatty acid oxidation complex subunit alpha and lipoxygenase) were highly induced in Mtvector especially in Rhizobia infection as compared to fungal infections. Proteins playing key role in nodulation, sulfur metabolism and pyrimidine biosynthesis were only induced with *S. meliloti* infection; proteolysis, chaperone activity, protease inhibitor (kunitz-type) and protein modification after *A. euteiches* infection, as well as proteins responsible for protein-protein interaction (14-3-3 like protein), ion binding (blue type copper domain) and NAD binding only induced after *G. intraradices* infections

(supplementary table 1). G proteins have been shown to play a key role in nodulation signalling (Choudhury et al., 2013), hence low induction of nodulation proteins in MtROP9i provides evidence to the interfered signal transduction process during root-rhizobia symbiosis.

2.4 Overview of protein induction via Heatmap

Overall, a comparative analysis of induced protein patterns considering all infections showed a distinctively lower number of total proteins induced in MtROP9i transgenic roots both for pathogenic and symbiotic infections compared to Mtvector roots. This indicates the responsive role of ROP GTPase MtROP9 protein during infection signalling whereby under conditions of hampered ROS synthesis, induction of early defense-related infection protein network mainly comprised of ROS-related enzymes was significantly affected, thereby compromising the first line of host defense (Fig. 2 and supplementary fig. 1a-c). The amount of total protein induction during rhizobia infection was stronger as compared to the fungal infections which hints to the fact that albeit rhizobia exists as a symbiont, it tends to portray some degree of pathogenic traits that well defines its evolutionally origin (supplementary fig. 2). Considering fungal infections, the induction pattern reached maximum at 5 hpi in Mtvector roots with significant reduction at 24 hpi, but with rhizobia infection, the induction reached maximum as early as at 3 hpi remaining constant even at a later timepoint (supplementary fig. 1a-c: minigraphs). The induction pattern in rhizobia infection relates to the complex host cellular events orchestrated that comprises a broad array of protein networks including the nodule-specific proteins that precede morphological and developmental changes meant to support the symbiotic process.

2.5 Defense-related proteins induced during symbiotic and pathogenic interactions

165 proteins were significantly induced during pathogenic and symbiotic interactions, which were classified as defense-related proteins. In pathogenic infections with *A. euteiches*, 64 proteins were induced in MtROP9i but with the majority being in Mtvector, where 101 proteins were induced. In symbiotic interactions with *S. meliloti*, 91 proteins were found to be induced of which 27 proteins were induced in MtROP9i and 65 proteins in Mtvector, while with *G. intraradices* infections, a total of 43 proteins were induced with 15 and 28 proteins induced in MtROP9i and Mtvector, respectively. The set of defense proteins identified especially in Mtvector comprised those induced during the early infection events associated with ROS production and hypersensitive response functions. In MtROP9i transgenic roots, majority of defense proteins induced comprised those involved in alternative/secondary defense pathways since the presence of ROS in the cells was compromised.

These include proteins involved in cell wall degradation such as endochitinases (33 fold at 5h) and polygalacturonase inhibitor (22 fold at 24h), protein degradation such as SGT1(77 fold at 3h) protein and proteolysis such as nectarin IV (10 fold 3h).

The induction of antioxidative defense products, ROS detoxification and degradation enzymes were previously reported (Schenkluhn et al., 2010). RBOH family proteins, which is a part of plasma membrane associate NADPH oxidase complex that regulates ROS production was highly induced especially at 3 h with *S. meliloti* infection.

RBOH play a crucial role in ROS generation during abiotic and biotic stresses (Dubiella et al., 2013) which can also act as a signalling intermediate in plants (Steffens et al., 2013). The RAC/ROP proteins shown to interact with a NADPH oxidase homologue hence regulating the ROS production is hereby re-affirmed by reduced induction pattern of RBOH in MtROP9i transgenic roots. Previously, we reported the knockdown of GTPase MtROP9 in *M. truncatula* resulting in hampered ROS regeneration and suppressed MtRBOH gene expression during microbial infections (Kiirika et al., 2012). With the current study, the induction of several RBOH proteins especially during symbiotic interactions with *S. meliloti* gives a clear indication of a stronger and prolonged ROS induction as opposed to pathogenic interactions, providing an elaborate background on the correlation that co-exists between the PR protein inductions and ROS production during systemic acquired resistance (SAR) in response to pathogen attack. Interestingly, a cytoplasmic redox protein nonexpressor of PR1 (NPR1) was induced only in Mtvector roots at 5 hpi after pathogenic infection with *A. euteiches*, implying that its induction was hampered with the knockdown of MtROP9. Furthermore, it was not induced in any of symbiotic infections (http://www.gelmap.de/380). NPR1 proteins function in sensing salicylic acid (SA) during microbial infection where it exists in the cytoplasm as an oligomer in its noninduced forms but reduced to its monomeric form after pathogen infection and translocated to the nucleus leading to induction of PR proteins (Rochon et al., 2006; Vlot et al., 2009). Plants produce high concentrations of SA during SAR both in infected and non-infected tissues that functions in the signalling of defense responses including induction of PR proteins (Oide et al., 2013). Presence of cytoplasmic ROS is shown to inhibit the action of NPR1 protein hence suppressing induction of PR proteins in *M. truncatula* seedlings infected with an incompatible bacteria *Pseudomonas syringae* (Peleg-Grossman et al., 2012). Our findings therefore, suggests that the suppressed induction of NPR1 protein at the early points of infections and particularly in Mtvector roots could be due to the high levels of ROS at the very early point of infection. ROS production was reported to be stronger and longer during symbiotic interactions with *S. meliloti* treatment on root cells than with pathogenic interactions with

Pseudomonas putida depicted by oxidative burst in the cytosol and not in the vacuoles confirming the essential role of ROS in establishment of symbiosis (Peleg-Grossman et al., 2012). Furthermore, the predominant induction of glutathione-S-transferase (GST) in *A. euteiches* infections indicates the activation of GST redox system responsible for primary antioxidative processes. Activation of several enzymes of oxidative defense including peroxidases, catalases, superoxide dismutase (Cu-Zn) as well as enzymes involved in ROS production and hypersensitive response induction such as harpin binding protein 1, hydroxyacylxylotathione hydrolase as well as perforin domain containing protein 1 were also detected.

A group of PR proteins of class 10, 1a, 4, 5a, Pprg2 and members of osmotin/thaumatin-like proteins of PR5a family were induced, exhibiting highest induction levels both at 3 hpi in Mtvector roots infected with *S. meliloti* and *A. euteiches*. However, for the osmotin/thaumatin-like proteins, the highest induction was found with the latter infection at 3hpi in MtROP9i transgenic roots. Moreover, additional PR-10-type proteins were detected annotated as abscisic acid responsive proteins (AB17s) mainly at 3 and 5 h in Mtvector roots after *A. euteiches* infection. Abscisic acid in *Arabidopsis* guard cells was shown to enhance levels of ROS (Pei et al., 2000). The meagre traces of ROS-related enzymes detected at later stages of infection may have been enhanced by the presence of abscisic acid in the infected cells whereof indirectly affirmed by the significant induction of abscisic acid responsive proteins. Induction of PR proteins occurred at advanced stages of infection representing a secondary line of host defense system especially in MtROP9i roots where the early molecular response characterized by ROS generation were compromised. Their induction was also reported earlier on with *A. euteiches* infections as the major component in the pathogen defense response of *M. truncatula* both at transcript and protein levels (Schenkluhn et al., 2010; Colditz et al., 2004, 2005 and 2007). A classification which provided cluster organization for proteins with similar biological activity or physiochemical properties and sequence homology divided the PR proteins into 17 classes PR1-PR17 (Fernandes et al., 2013). The role PR10 protein in particular is not well understood since they are constitutively expressed in plants particularly in response to pathogenic and environmental stresses. They are suggested to present a protective role in plants as well as in general plant development (Fernandes et al., 2013). The PR-5 proteins share sequence homologies with thaumatin, a protein isolated from *Thaumatococcus daniellii*, hence its members are referred to as thaumatin-like proteins (TLPs). TLPs are classified into three groups comprising those induced in response to pathogen infection, osmotic stress the so-called osmotins and the antifungal proteins (Breiteneder, 2004). They

have also been reported to be induced after pathogenic infection and presenting the antifungal and antioomycetes activity (Fernandes et al., 2013).

The major latex proteins (MLP) are a family of proteins whose function is unknown. They were detected at 3 and 5hpi in *A. euteiches* and *G. intraradices* infections only but interestingly not with *S. meliloti* infection suggesting their expression specificity to fungal infections. MLPs are implicated in plant development as well as induced during plant response to stimuli particularly during pathogen defense responses. Based on sequence similarity, they are classified as members of Bet v 1 protein superfamily 2 with 24 proteins identified in Arabidopsis only. In soybean for example, both MLPs and PR10 are known to coexist, hence suggested to play similar functional role as they also share a similar sequence homology (Betsy et al., 2009).

2.6 Stress-related proteins induced during symbiotic and pathogenic interactions

316 induced proteins were classified as stress-related proteins from all the three infections. 132 proteins were induced after *A. euteiches* infection, with 51 and 81 proteins induced in MtROP9i and Mtvector, respectively. The protein P21 kinase inhibitor, a potent cyclin-dependent kinase inhibitor (CDKI) was highly induced (50 fold) and is involved in stress reaction of plants by deregulation of cell proliferation controlling growth pathways cell-cycle regulatory and integration of developmental signals in the cell machinery. CDKI functions by inhibiting the activity of CDK2 or CDK1 complexes (De Veylder et al., 2001). A glycine-rich RNA-binding, abscisic acid-inducible protein and cold shock protein-1 were induced after infection and are involved in post-transcriptional regulation of gene expression in plants under biotic or abiotic stresses. Plant glycine-rich proteins are characterized based on their general structure and categorized into classes I-V considering the arrangement of the glycine repeats and conserved motifs. They function as RNA chaperones by binding to target mRNA leading to destabilization of the over-stabilized secondary structures. Hence, they facilitate translation even under conditions of plant stress in the course of infection development, and cell wall fortification through polymerization process by cross-linking (Mangeon, 2010). Exposure of *A. thaliana* plants to cold stress conditions ubiquitously expressed glycine-rich RNA-binding proteins in various organs including stem, roots, leaves flowers and siliques (Kim et al., 2005). Thioredoxins that function in regulation of redox status in the cell were highly induced in Mtvector at 3 hpi as compared to MtROP9i roots after *A. euteiches* infection. Their induction may have been triggered in order to counteract the pathogen-orchestrated prelude of ROS synthesis that could result to scavenging mechanisms to the host cells during the early phases of pathogenic infection. Consequently, their

insignificant induction in MtROP9i transgenic roots clearly depicts the inconspicuous generation of ROS or ROS-related enzymes whose induction levels could not surmount to a coordinated synthesis of the redox regulatory enzymatic system comprising thioredoxin in the host cell. Thioredoxins have been shown to possess a fundamental role in plant tolerance to oxidative stress by presenting a reducing power to reductases detoxifying lipid hydroperoxides as well as modification or repair of oxidized proteins. They have also been reported to play a role in regulation of signalling pathways as well as the ROS scavenging mechanisms in plants (Dos Santos and Rey., 2009).

In symbiotic interactions, 92 proteins were significantly induced and were classified as stress-related proteins. 34 and 58 proteins were induced in MtROP9i and Mtvector, respectively, after *S. meliloti* infections. The prohibitin protein was highly induced which was also reported previously in *M. truncatula* mitochondrial fractions by Dubinin et al. (2011) and *M. truncatula* cells by Trapphoff et al. (2009) after inoculation with virulent spores of oomycete pathogen *A. euteiches*. Recent findings indicate that prohibitins are involved in mediating stress tolerance (abiotic stress, pathogen infection and elicitor signalling) as well as triggering retrograde signals in response to mitochondrial dysfunction (Aken et al., 2010). The peroxidases involved in scavenging of peroxide and detoxification of ROS were also induced. After *G. intraradices* infections, 92 proteins were induced, where 42 and 50 proteins were found in MtROP9i and Mtvector, respectively. Among the induced proteins, clathrin assembly protein (At4g32285) involved in the formation of vesicles in the cytoplasm for intracellular protein trafficking was highly induced.

2.7 Signal-transduction proteins induced during symbiotic and pathogenic interactions

183 proteins were induced both in symbiotic and pathogenic interactions which function in signal transduction pathways. Infection with *S. meliloti* resulted in induction of 95 proteins with 31 and 64 proteins found induced in MtROP9i and Mtvector, respectively. After *G. intraradices* infection, 69 proteins were induced with 23 and 46 proteins induced on MtROP9i and Mtvector, respectively. In pathogenic interactions, 109 proteins were found to be induced where 33 and 76 proteins were induced in MtROP9i and Mtvector, respectively. Over all, majority of induced proteins in the signal transduction category were found in *A. euteiches* inductions but levels of inductions for individual proteins was higher in *S. meliloti* infection.

The nodule-specific cysteine-rich peptide 96 (induced 78 fold at 5 hpi) which plays a key role in the early nodulation signalling was induced only in *S. meliloti* infection. Other proteins involved in GTP binding/GTPase activity were highly induced in Mtvector roots but exhibited low inductions in

MtROP9i. Induction of GTP-binding protein family constituting the GTP binding nuclear protein Ran/TC4/Ran-a1 protein, guanine nucleotide-binding protein subunit beta-like protein were not significantly induced in MtROP9i as compared to Mtvector. GTP binding proteins are involved in diverse cellular processes in plants where they cycle between active (GTP-bound) and inactive (GDP-bound) states and interacts with other protein partners functioning as important molecular switches essential for numerous cellular functions in plants including signal transduction and transport pathways (Poraty-Gavra et al., 2013). Suppressed induction of GTP binding proteins in MtROP9i transgenic roots confirms the successful knockdown of MtROP9 protein. The silencing was specific for this protein as it exhibits little sequence conservation with other GTPases, hence eliminating any chances of cross-silencing for other GTP binding proteins. Therefore, the observed minimal accumulation of proteins responsible for ROS production and detoxification even in the MtROP9i silenced roots could also be due to RBOH interacting with other non-target GTPases actively present in the cytosol.

Calmodulin (CaM) 1, 2, 8 and CaM binding proteins were induced in the category of calcium second messengers, which constitutes proteins involved in the initial plant-microbe interactions. CaM mediates the calcium-dependent signalling by functioning as a decoder for the Ca^{2+} signatures during signal transduction especially at the early points of infection (Bender et al., 2013). Induction of CaM depicts progressive colonization of the tissue by the microbe thereby eliciting the secondary line of defense barrier. Calreticulin protein that functions in controlling plant defense by regulating the concentration of Ca^{2+} ions in the cell during signalling pathways as well as acting as molecular chaperone (Qiu et al., 2012) was induced only in Mtvector roots, particularly in fungal infections and suppressed in MtROP9i. Interestingly, the CaM and calreticulin proteins were highly induced in the Mtvector roots which also had concomitant high induction of ROS-related enzymes, suggesting an intimate interconnection between ROS and calcium signatures. Both signals most likely represent a cross-talk that is constituted to modulate downstream nuclear activity resulting in induction of pathogenic or symbiotic specific protein networks. The membrane-bound NADPH oxidase that is activated by ROP GTPases to generate ROS is shown to have a cytosolic N-terminal region that contains two EF-hand domains, suggesting that their activation is dependent on binding of Ca^{2+}, hence the enzyme is thought to behave like a Ca^{2+} sensor. The NADPH oxidase is also activated by phosphorylation of the N-terminal serine residues by the Ca^{2+}- dependent protein kinase (Kaboyashi et al., 2007), suggesting the complex structure of NADPH oxidase protein interacting partners of which ROP GTPase is a predominant part of, aimed at mediating ROS production at the plasma membrane.

Calreticulin was recently shown to confer resistance against oomycete pathogen phytopthora infestans in *Nicotiana benthamiana* (Matsukawa et al., 2013). Induction of the translationally controlled tumor protein homologue (TCTP) was low in MtROP9i as compared to Mtvector, but with the highest induction in *S. meliloti* infection. TCTP is shown to be a Ca^{2+} -binding protein ubiquitously expressed in all eukaryotic cells (Zhang et al., 2013). In our previous studies, we reported its high induction at the transcript level after elicitor-induced signalling in tomato against soilborne bacteria *Ralstonia solanacearum* (Kiirika et al., 2013). Currently, evidence is emerging on its involvement in negative regulation of hypersensitive reaction as shown in *N. benthamiana* plants challenged with *R. solanacearum* (Gupta et al., 2013). Suppressed expression of TCTP in MtROP9i transgenic lines indicates the reduced potential of ROS present in the cell that would indirectly trigger the downstream induction of TCTP. Recently, two modes of action of TCTP cytoprotective activity have been suggested, where it is said to act as a sequester of Ca^{2+} hampering programmed cell death (PCD) by reducing levels of Ca^{2+} in the cytosol. It could also interact with other cytosolic membranous proteins of the cytosolic PCD machinery, thereby mitigating the cell death progression (Hoepflinger et al., 2013). In addition, high induction of caffeic acid 3-O-methyltransferase (CCOMT), a protein involved in conversion of caffeoyl-CoA to synapoyl-CoA, an intermediate in lignification in cells under stressed condition (Kosova et al., 2013) was induced particularly with the fungal infections while exhibiting high inductions in Mtvector roots. Typical for fungal infection, the cell wall entry by the developing fungal infection peg and the hypopodia transcending the membranes trigger the host membrane re-adjustments and the downstream signalling cascade characterized by ROS production targeted towards restricting further infection to the neighboring cells. The preceding scavenging mechanisms by high ROS in cells results to host's cell wall strengthening involving structural reinforcement of the cell walls and increased lignin polymer content (Dennes et al., 2012; Tipathy and Oelmüller, 2012). Taken together, the mechanisms of fungal invasion and ROS scavenging to the cell triggers CCOMT synthesis to function putatively as a compensatory response by enhancing lignin formation so as to reinforce the cell wall and maintain its functional integrity.

2.8 Energy metabolism proteins induced during symbiotic and pathogenic interactions

A total of 590 proteins were found to be significantly induced that play a crucial role in energy metabolism in plants. In symbiotic infections, 189 proteins of which 81 and 107 proteins were induced in MtROP9i and Mtvector, respectively, after *S. meliloti* infection. 170 proteins were significantly induced after *G. intraradices* infection of which 81 and 87 proteins were induced in MtROP9i and

Mtvector respectively. After pathogenic infection with *A. euteiches*, 233 proteins were found to be induced with 76 and 157 proteins in MtROP9i and Mtvector, respectively. The highest amount of protein induction was after *A. euteiches* infection, albeit the amount of protein induction was highest after *S. meliloti* infection. In the course of pathogenic infection, energy metabolism is a prerequisite for synthesis of defense products and compensation of cellular losses during the infection process. Proteins involved in energy regulatory processes in the cell were induced including those that function in carbohydrate metabolism (e.g. sucrose synthase, aconitate hydratase and beta-glucosidase G1), pyruvate and TCA cycle metabolism (e.g. NADP-dependent malic enzyme and citrate synthase), oxidative phosphorylation (e.g. NADH dehydrogenase [ubiquinone] 1 alpha and malate dehydrogenase), glycolysis (e.g. glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase and Aldehyde dehydrogenase) and reductive pentose phosphate pathway (e.g. transketolase 1, Glucose-6-phosphate 1-dehydrogenase and transaldolase). ROS production generally occurs as a byproduct of aerobic metabolism tightly controlled by scavenging pathways and may act as second messenger for various activities downstream in the cell such as altering the redox balance. Redox signals orchestrates energy metabolism response in order to compensate for utilization of cellular products in the cell to counter the prevailing stress cues (Bhattachajee et al., 2011). This could explain why the protein induction pattern in the category of energy metabolism was low in MtROP9i transgenic roots where the potential for ROS synthesis was hampered.

3. Conclusion

The early molecular events in the cell upon microbial infection comprises ROS defense signalling that often culminates to PCD. ROS generation events are fundamentally orchestrated by the activation of RBOH upon interaction with the small GTPases. However, when the components supporting ROS synthesis are compromised, the host cell activates alternative defense pathways by synthesis of defense enzymes that does not form part of ROS defense system. Therefore, from the current investigations, we have shown that silencing of small GTPase MtROP9 in *M. truncatula* roots results in significant suppression of ROS-related enzymes at the early points of infection both for symbiotic and pathogenic interactions and a concomitant activation of alternative defense pathways by the host cell to counteract the infection progress. The induction of ROS-related enzymes such as RBOH 1/B, glutathione-S-transferase, peroxidases, catalases, superoxide dismutase was suppressed in MtROP9i transgenic roots as compared to Mtvector roots, but proteins involved in alternative pathways such as cell wall degradation, chitin hydrolysis and proteolysis, PR proteins were highly induced. This implies that the

RBOH activity responsible for oxidative burst during microbial infection was affected. Other proteins whose induction was affected also were involved in signal transduction pathways, stress response, antioxidative, peroxidase and chaperone activities. Proteins involved in energy metabolism exhibited minimal differential induction in MtROP9i as compared with Mtvector roots, implying that enzymes responsible for energy metabolism which is a prerequisite for the progress of infection were not significantly affected. The total amount of protein induction was highest after *S. meliloti* infection and comparable to *A. euteiches* infection confirming the pathogenic evolutionary character of rhizobia bacteria that is said to have recruited the protein components supporting symbiosis, hence becoming a symbiont (Willems and Collins, 1993). Despite the overlapping similarities existing between pathogenic and symbiotic interactions, the host plant has to maintain specificity such that it activates response cues that are either meant to promote or desist the interaction. This process consists of broad array of protein networks forming central dynamics in the cell of which when analyzed give huge proteome datasets. The new heatmap-GelMap module presented allowed systematic and easy visualization of all induced proteins displaying also their physiological functions in the cell. To this end, the elaborate protein induction evaluation upon symbiotic and pathogenic interactions reported herein, depicts the essential role of small GTPase MtROP9 during the early ROS defense signalling in plants.

4. Materials and methods

Construction of RNAi Vector

Transgenic MtROP9i roots were produced using the binary vector pK7GWIWG2(II)::DsRED (kindly provided by E. Limpens; Limpens et al., 2005) containing the gene for red fluorescent marker DsRED1. The vector was modified by insertion of two sequence cassettes (in the sense-antisense direction) encoding parts of the putative effector (G2) and GTPase (G3) domains of the MsRac1 ortholog MtROP9 (TC173331; GenBank accession no. AF498359). Binary vectors for gene knockdown by RNAi were constructed using the Gateway technology (Invitrogen Life Technologies). Gene-specific oligonucleotides (ROP9attb1_for, 5′-attB1 GTGTTACTGTTGGTGATG-3′;ROP9attb2_rev,5′-attB2-ACGCCTTCACGTTCTCC-3′) with attached attB adapters were obtained from the *Medicago sativa* MsRac1 sequence (GenBank accession no. AJ251210; Schiene et al., 2000). Using these oligonucleotides, amplification of a 461-bp fragment from *Medicago truncatula* cDNA was carried out and the PCR products purified using the QIAquick PCR purification kit (Qiagen) and cloned into the pDONR221 donor vector (Invitrogen). In the second cloning step, the inserts were

transferred into the Gateway-compatible binary vectors mentioned above followed by transformation into *Agrobacterium rhizogenes* strain ArquaI (Quandt et al., 1993) using standard methods. The presence of the MtROP9 sequence fragments and the sense-antisense orientation of the cloned fragments in the T-DNA were confirmed by sequencing the construct, while nonmodified binary vectors were transformed into *A. rhizogenes* ArquaI as a control.

Generation of transgenic roots and infection assays

M. truncatula MtROP9i and Mtvector transgenic roots were generated according to the Boisson-Dernier et al. (2001) using *M. truncatula* (Jemalong A17) wild-type plantlets as described before (Colditz et al., 2007). *M. truncatula* composite plants with roots transformed by *A. rhizogenes* were cultured stably on M medium (Bécard and Fortin, 1988) containing 25 mg L21 kanamycin for selection and decreasing concentrations of 350 to 0 mg L21 ticarcillin disodium/clavulanate potassium (Duchefa) to stop growth of *A. rhizogenes*. Twelve individual populations of composite plants with MtROP9i and Mtvector transgenic roots were generated independently via *A. rhizogenes* transformation, containing at least 200 plants each. Populations were cultured on M medium and kept in the growth chambers at 22°C, 65% humidity, 16-h photoperiod at 220 µE m^{-2} s^{-1}). Inoculation with *A. euteiches* (ATCC 201684) was carried out as described before (Colditz et al., 2007). Each transgenic root population was infected with 500 mL of lake water containing 250,000 *A. euteiches* vital zoospores. Inoculation with *G. intraradices* was performed using commercially available inoculums (Granular AMF inoculum; BIORIZE). Infection with *Sinorhizobium meliloti* wild-type strain Rm2011 was performed as described previously (Schenkluhn et al., 2010).

Protein isolation, 2D IEF SDS-PAGE and gel evaluation

Phenol extraction of total protein from the cells was carried out as described previously (Colditz et al. 2004, 2005, 2009). For IEF, 3 mg of protein was diluted with 350 µl of rehydration buffer, consisting of 8 M urea, 2% (wt/vol) CHAPS, 100 mM dithiothreitol, 0.5% (vol/vol) IPG buffer for correspondent p*I* range (pH 3–11 nonlinear [NL]; Amersham Pharmacia Biotech, Uppsala, Sweden), and a trace of bromphenol blue. 2D IEF SDS-PAGE was performed for the three infections and at four timepoints of harvesting by combining the IEF strips (IPGphor system) with a sodium dodecyl sulfate–tricine gel electrophoresis (Protean II XL, 20 by 20 cm; BioRad, Richmond, CA, U.S.A.) as already described (Colditz et al. 2005). Gels were stained with 0.1% (wt/vol) Coomassie Brilliant Blue (BioRad) overnight and scanned on a UMAX Power Look III Scanner (UMAX Technologies, Fremont, CA, U.S.A.). Gels were evaluated using Delta 2D software, version 4.0 (Decodon, Greifswald, Germany) with three replicates per group (1, 3, 5 and 24 hpi). Spots detection was done automatically and

occasionally corrected manually. In gel normalization was performed using the Delta 2D software for the overlays of three replicate gels each. Spots with a relative spot volume of less than 0.05% were deleted and the significant abundance of spots between MtROP9i and Mtvector groups was determined using Student's *t*-test (confidence interval ≥95%) based on the relative spot volume.

Mass spectrometry and creation of heatmap-Gelmap

Protein spots of 1.4mm diameter were cut from Coomassie stained gels using a GelPal Protein Excision manual spot picker (Genetix, Great Britain) and in-gel digested with Trypsin as described by Klodmann et al. (2010). Tryptic peptides were further analyzed by nanoHPLC (Proxeon, Thermo Scientific) coupled to electrospray ionization quadrupole time of flight MS (micrOQTOF Q II, Bruker Daltonics), using all settings and parameters as described previously (Klodmann et al., 2011). Data processing and protein identification was carried out with ProteinScape 2.0 (Bruker Daltonics) and the MASCOT search engine querying three *Medicago*-specific protein databases [*Mt3.5 ProteinSeq, NCBI Medicago truncatula protein, and Mtf*(*asta*)2] available at the LegProt db (Lei et al., 2011) as well as *SwissProt*, using the following parameters: trypsin/P; one missed cleavage allowed; fixed modifications: carbamidomethylation (C), variable modifications: acetylation (N) and oxidation (M); precursor ion mass tolerance, 30 ppm; peptide score >24; charges 1C, 2C, 3C. Protein and peptide assessments with MASCOT scores above 25 were considered. Heatmaps were created using the total significant induction (≥1.5 fold) of proteins per physiological function as annotated via *Swissprot* . Induction of proteins with similar functions was clustered and presented as values, which were filtered and displayed on a matrix. Colour-coding scheme ranging from dark red (high induction) to light red (low induction) was applied. Heatmap was linked with the reference GelMap platform (Senkler and Braun, 2012). A Heatmap image (.jpg) was assigned *x*- and *y*-coordinates to specifically allocate total inductions of each protein category into a corresponding colour-code range on a Heatmap matrix (Supplementary material figure 5). An Excel (Microsoft) file containing all protein information including MS/MS results and the corresponding Heatmap image (.jpg) were then imported into GelMap portal. Information on GelMap creation can be accessed at http://www.gelmap.de/howto.

5. Acknowledgements

We thank Michael Senkler, Institute of Plant Genetics, LUH Hannover, for assistance with the creation of the *Medicago truncatula* Heatmap-GelMap. We further thank Jennifer Klodmann for fruitful discussions and Holger Eubel, Institute for Plant Genetics, LUH Hannover, for support with MS analyses. We acknowledge support by Deutsche Forschungsgemeinschaft.

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Fig 1a. MtROP9i and V. control with *S. meliloti* infection

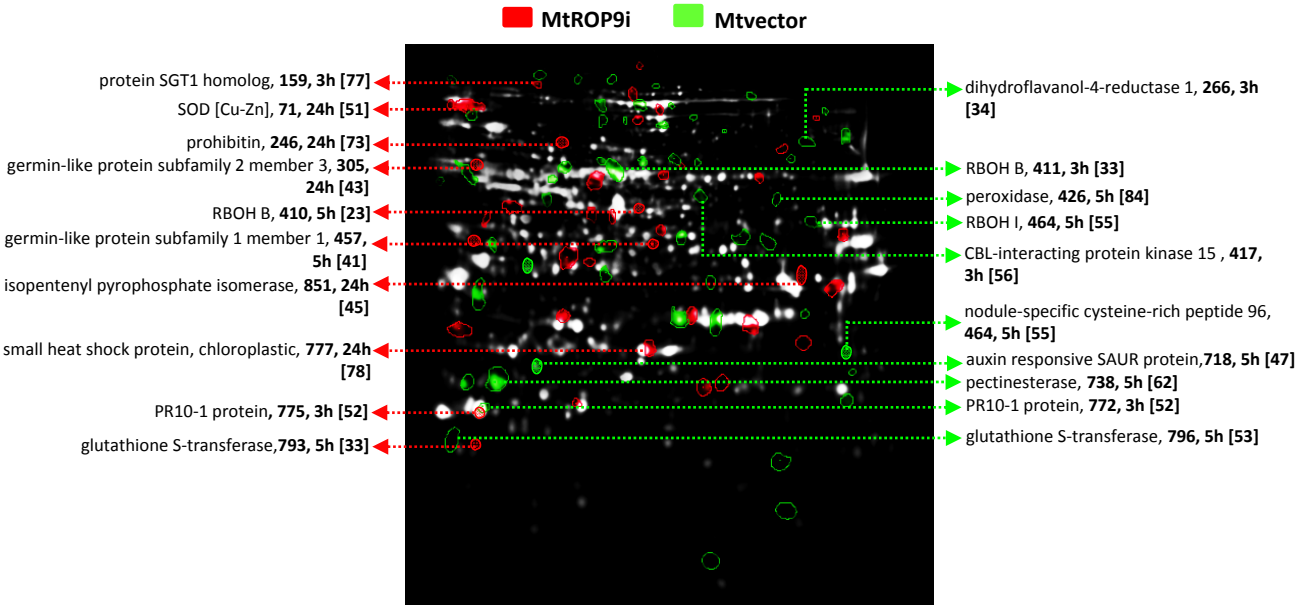


Fig 1b. MtROP9i and V. control with *G. intraradices* infection

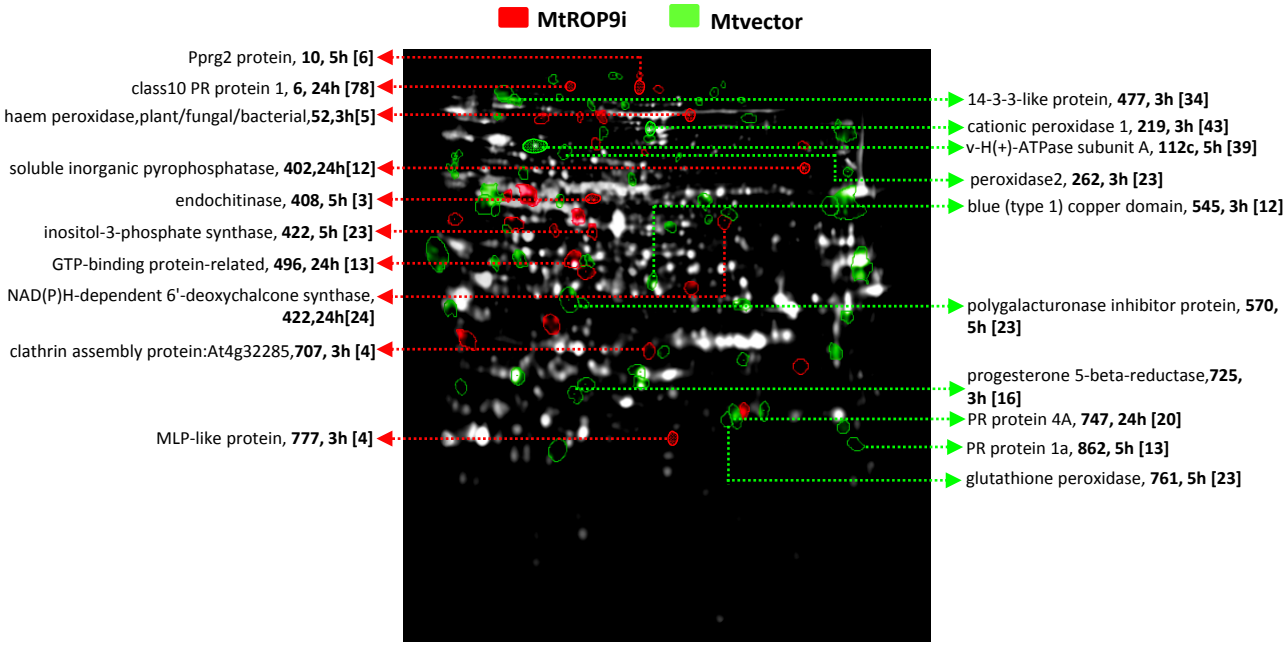


Fig 1c. MtROP9i and V. control with *A. euteiches* infection

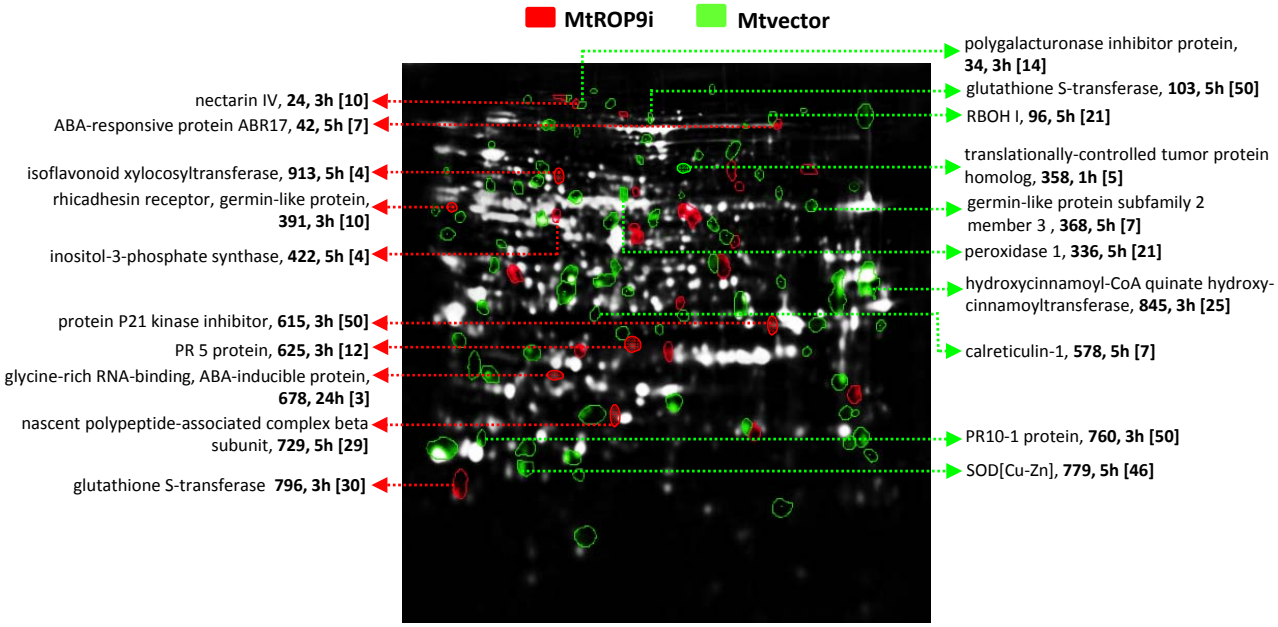


Fig 1. Proteins of high abundance in MtROP9i (red spots) and Mtvector (green spots) after *S. meliloti* (a), *G. intraradices* (b) and *A. euteiches* (c) infections for the predominant physiological categories defense response, stress response, signal transduction, secondary metabolite biosynthesis and transport. Proteins of equal abundance appear as white spots. The protein name, spot number, hours post inoculation (h) is given per protein as well as the level of abundance based on >1.5-fold, shown in square brackets.

Fig. 2 The heatmaps for *S. meliloti*, *A. euteiches* and *G. intraradices*

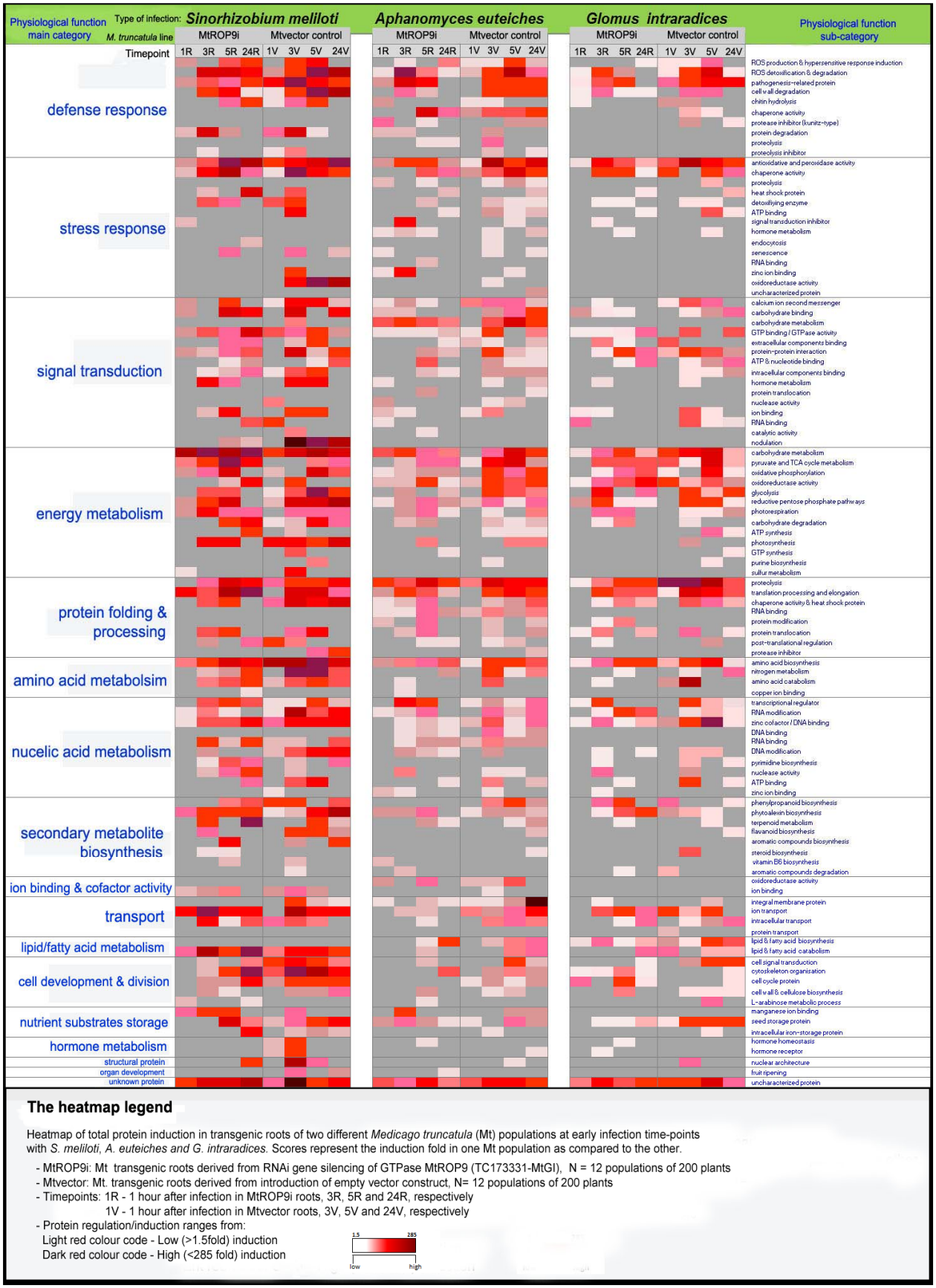


Fig. 1a - *S. meliloti*

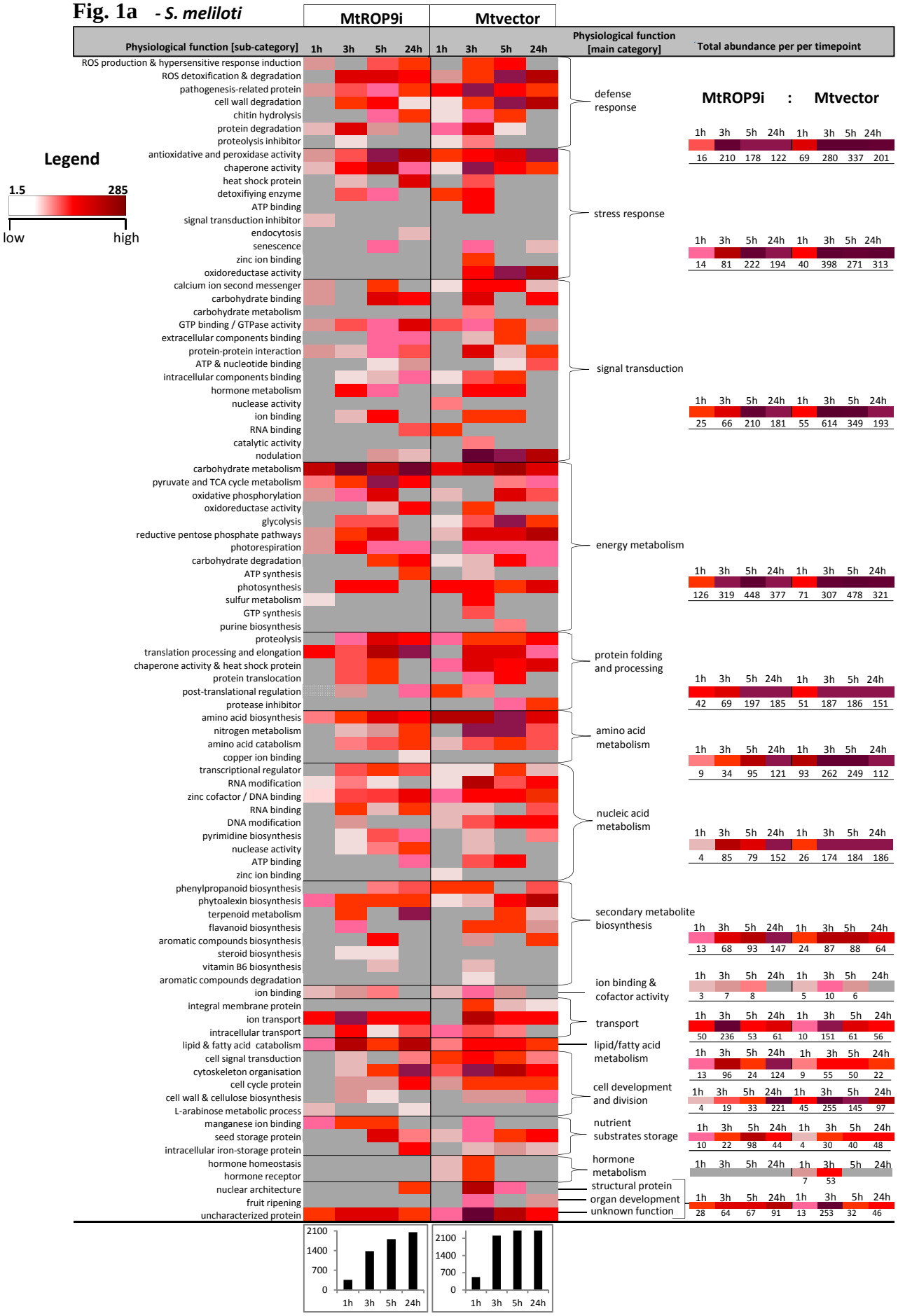


Fig. 1b - ***A. euteiches***

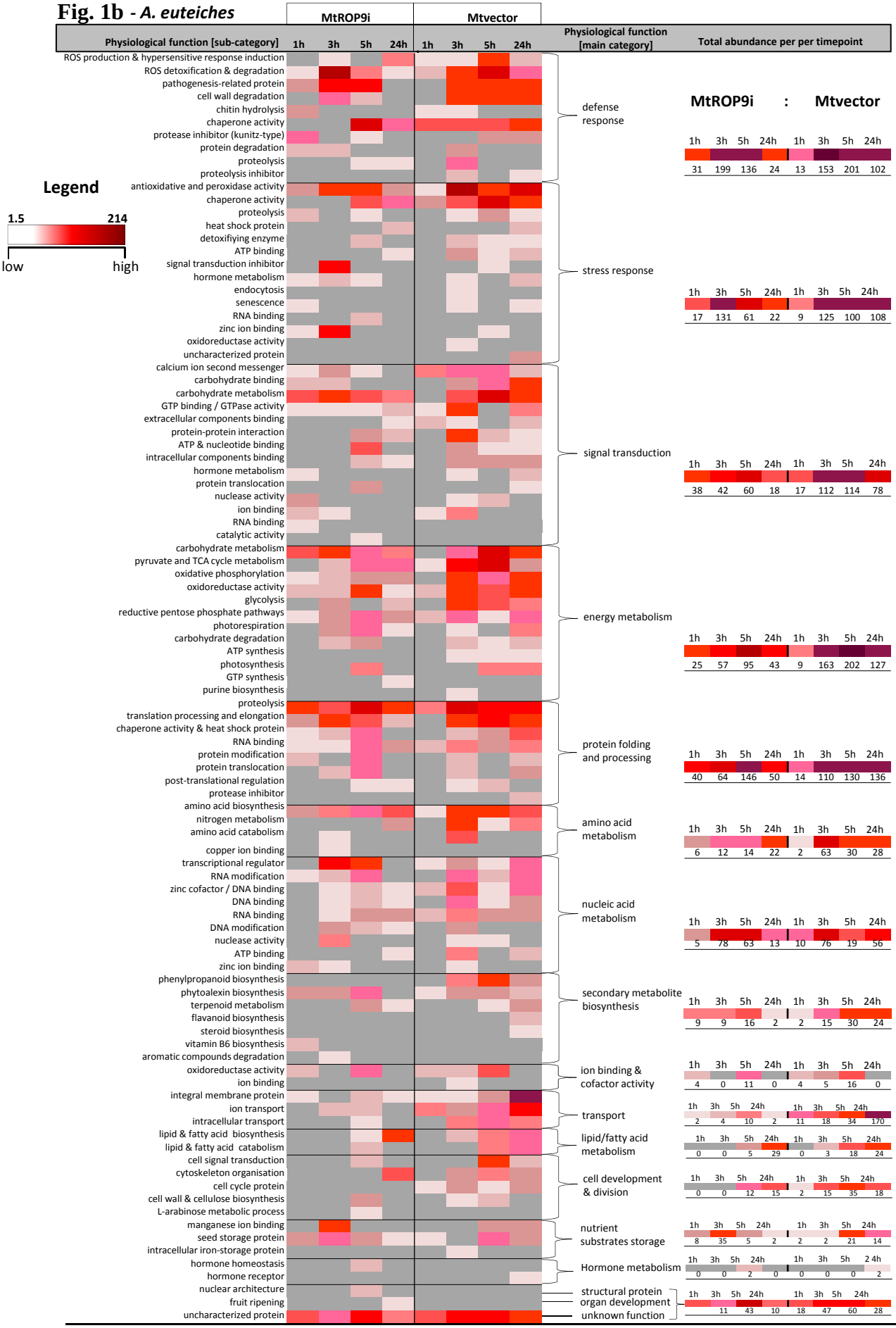
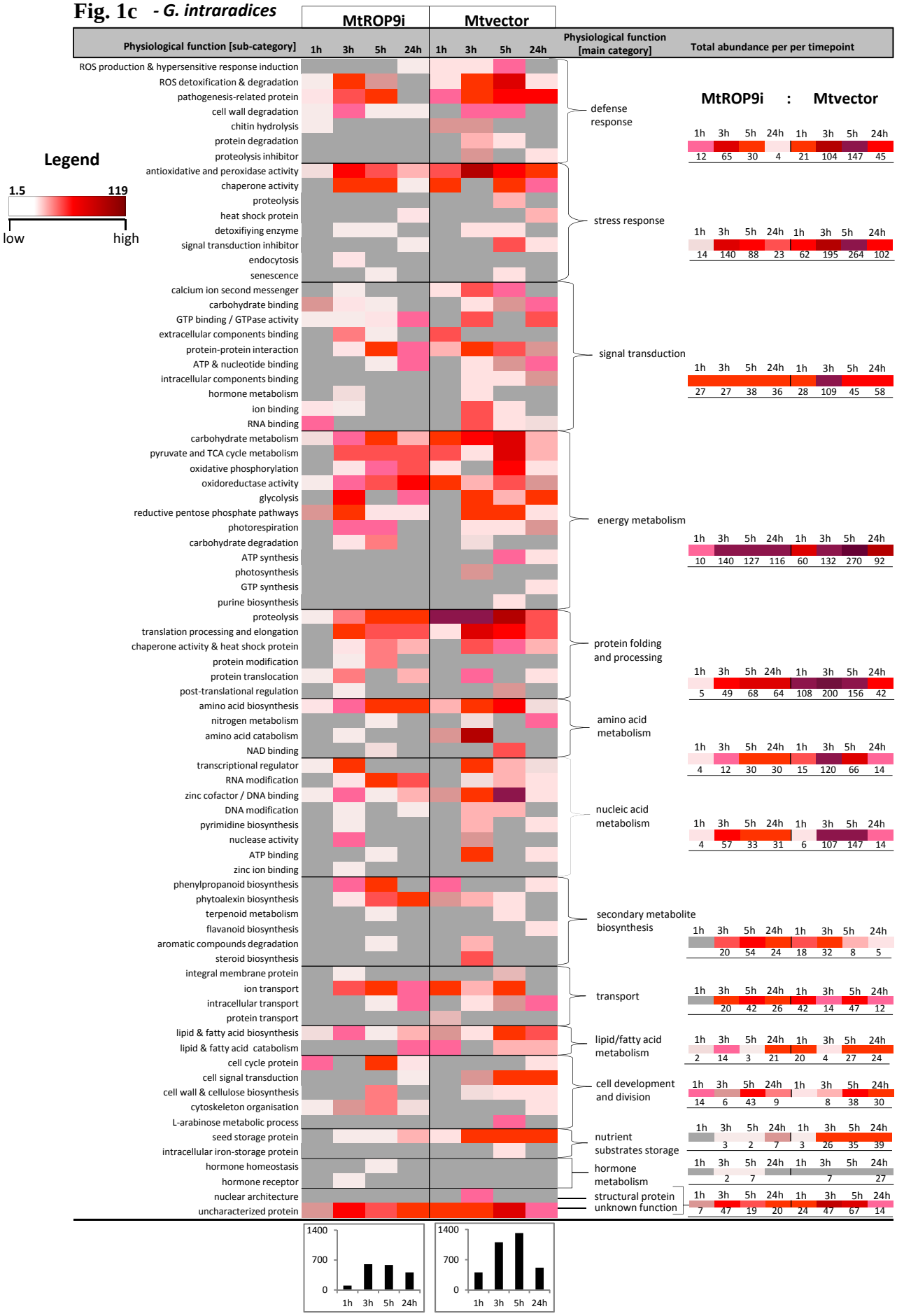


Fig. 1c - *G. intraradices*



A. euteuteiches

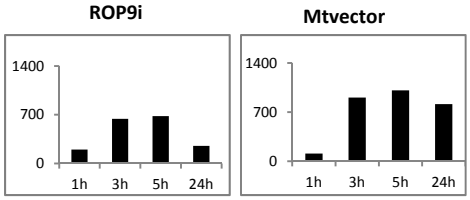


Fig. 2a - ***S. meliloti***

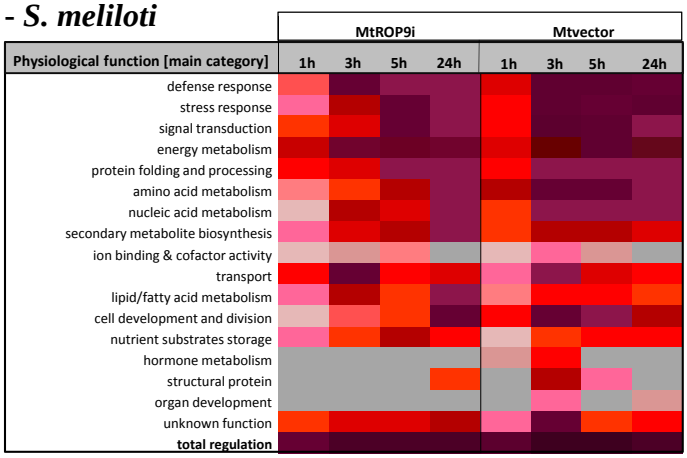


Fig. 2b - ***A. euteiches***

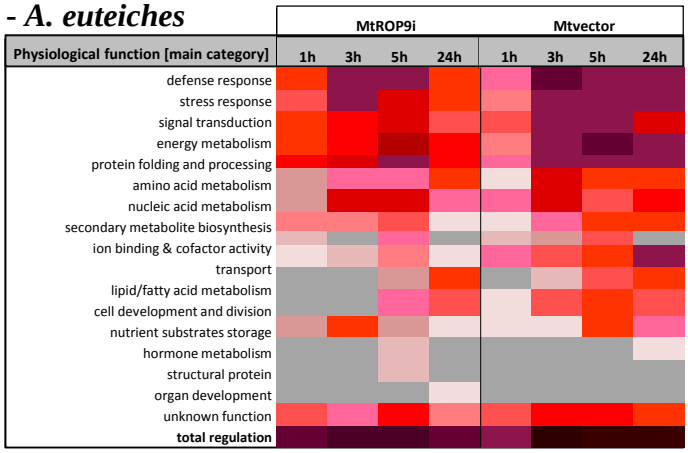
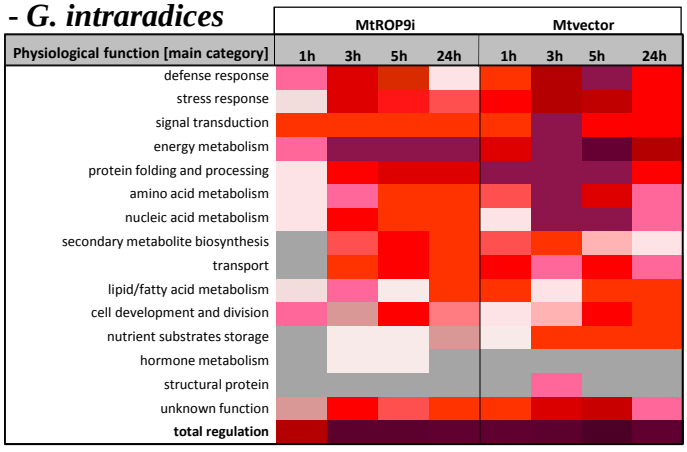


Fig. 2c - ***G. intraradices***



Supplementary figures 1 and 2: Protein induction patterns of *M. truncatula* MtROP9i and Mtvector roots after *S. meliloti* (1a), *A. euteiches* (1b) and *G. intraradices* (1c) infection visualized on a Heatmap. The heatmap shows distinct proteome subsets created from total induction values of all identified proteins classified into different physiological functions. The colour intensity potrayed as a shift in gradient from light red to deep red represents the relative changes from a minimum to a maximum value allowing easy spotting of trends or standouts in each infection at a given time after infection. The total induction for each category is shown on the right and the minigraphs for the trend of total induction over 1, 3, 5 and 24 hpi is shown at the bottom. **Fig 2: Heatmap of total protein regulation in each physiological category over the four timepoints for *S. meliloti* (2a), *A. euteiches* (2b) and *G. intraradices* (2c).**

Table 1. Protein categories induced only in specific infections

Physiological function (main category	Physiological function (sub-category)
Sinorhizobium meliloti	
secondary metabolite biosynthesis	aromatic compounds biosynthesis
signal transduction	hormone metabolism (auxin responsive SAUR protein)
	nodulation
nucleic acid metabolism	pyrimidine biosynthesis
energy metabolism	sulfur metabolism
	pyruvate and TCA cycle metabolism (malate dehydrogenase)
Aphanomyces euteiches	
defense response	chaperone activity
	protease inhibitor (kunitz-type)
	proteolysis
ion binding & cofactor activity	oxidoreductase activity
lipid/fatty acid metabolism	lipid & fatty acid biosynthesis
protein folding & processing	protein modification
	RNA binding
signal transduction	protein translocation
stress response	hormone metabolism
Glomus intraradices	
protein folding and processing	protein modification
signal transduction	protein-protein interaction(14-3-3-like protein)
	ion binding(blue (Type 1) copper domain)
	intracellular components binding (Ran binding protein)
stress response	heat shock protein(stromal 70 kDa heat shock-related protein
energy metabolism	oxidative phosphorylation(ATP synthase subunit delta)
lipid/fatty acid metabolism	lipid & fatty acid biosynthesis
amino acid metabolism	NAD binding
transport	protein transport

Table 2. List of proteins with highest induction in abundance for selected physiological categories

Spot No.	hpi	Protein name	Physiological function	Physiological function sub-category	Diff. ab.	Accession No.
			MtROP9i_*A. euteiches*			
796	3R	glutathione S-transferase GST	defense response	ROS detoxification & degradation	30	MTR_5g090910
729	5R	nascent polypeptide-associated complex subunit beta	defense response	chaperone activity	29	Medtr3g020520.1
625	3R	pathogenesis-related thaumatin-like protein	defense response	pathogenesis-related protein	12	Medtr5g010640.1
24	3R	nectarin IV	defense response	proteolysis	10	Q3KU27_NICLS
75	5R	ABA-responsive protein ABR17	defense response	pathogenesis-related protein	7	ABR17_PEA
615	3R	protein P21 kinase inhibitor	stress response	signal transduction inhibitor	50	P21_SOYBN
223	3R	cold shock protein-1	stress response	zinc ion binding	50	Q8LPA7_WHEAT
772	5R	thioredoxin fold	stress response	antioxidative and peroxidase activity	4	Q06H32_ARAHY
678	24R	glycine-rich RNA-binding, abscisic acid-inducible protein	stress response	RNA binding	3	GRPA_MAIZE
285	3R	peroxidase 1	stress response	antioxidative and peroxidase activity	3	Q9XFL3_PHAVU
755	5R	BiP	signal transduction	ATP & nucleotide binding	16	Q587K1_SOYBN
391	3R	rhicadhesin receptor, germin-like protein	signal transduction	carbohydrate metabolism	9	Medtr7g111240.1
22	3R	monocopper oxidase-like protein SKU5	signal transduction	ion binding	5	Medtr4g101650.1
422	5R	inositol-3-phosphate synthase	signal transduction	protein-protein interaction	4	INO1_NICPA
271	24R	receptor-like protein kinase	signal transduction	protein-protein interaction	3	MTR_3g009050
913	5R	isoflavonoid xylocosyltransferase	secondary metabolite biosynthesis	phytoalexin biosynthesis	4	Q7XZD0_GLYEC
379	1R	VDAC1.3	transport	ion transport	3	TC179231
			Mtvector_*A. euteiches*			
760	3V	PR10-1 protein	defense response	pathogenesis-related protein	50	MTR_2g035100
103	5V	glutathione S-transferase	defense response	ROS detoxification & degradation	50	GSTF5_ARATH
779	5V	SOD[Cu-Zn]	defense response	ROS detoxification & degradation	46	SODC_PEA
96	5v	RBOH I	defense response	ROS production & hypersensitive response induction	21	RBOHI_ARATH
34	3V	polygalacturonase inhibitor protein	defense response	cell wall degradation	14	MTR_7g023690
845	3V	peroxidase 1	stress response	antioxidative and peroxidase activity	25	PER1_ARATH
29	3V	thioredoxin h	stress response	antioxidative and peroxidase activity	8	A1BLP6_MEDTR
305	5V	peroxidase pxdC	stress response	antioxidative and peroxidase activity	7	Q40366_MEDSA
572	3V	HSP70-related protein (BiP)	stress response	chaperone activity	6	Q587K1_SOYBN
391	24V	peroxidase2	stress response	antioxidative and peroxidase activity	5	O24080_MEDSA
29	3V	putative membrane protein ycf1	signal transduction	protein-protein interaction	8	YCF1_LOBMA
578	5V	calreticulin-1	signal transduction	calcium ion second messenger	7	A0A762_SOYBN
368	5V	germin-like protein subfamily 2 member 3	signal transduction	carbohydrate binding	7	GL23_ARATH
556	3V	endoplasmic reticulum HSC70-cognate binding protein	signal transduction	ATP & nucleotide binding	6	O22639_SOYBN
358	1V	TCTP(translationally-controlled tumor protein homolog)	signal transduction	calcium ion second messenger	5	TCTP_MEDSA
336	5v	hydroxycinnamoyl-CoA quinate hydroxycinnamoyltransferase	secondary metabolite biosynthesis	phenylpropanoid biosynthesis	21	TC194348
391	24V	short-chain dehydrogenase TIC 32, chloroplastic	transport	intracellular transport	5	TIC32_ARATH
			MtROP9i_*S. meliloti*			
159	3R	protein SGT1 homolog	defense response	protein degradation	77	SGT1_ORYSJ
71	24R	SOD [Cu-Zn]	defense response	ROS detoxification & degradation	51	MTR_7g114240
793	5R	glutathione S-transferase	defense response	ROS detoxification & degradation	33	GSTUM_ARATH
775	3R	PR10-1 protein	defense response	pathogenesis-related protein	52	MTR_2g035100
410	5R	RBOH B	defense response	ROS production & hypersensitive response induction	33	RBOHB_SOLTU
246	24R	prohibitin	stress response	chaperone activity	73	Medtr3g008250.1
777	24R	small heat shock protein, chloroplastic	stress response	heat shock protein	78	HS21C_Wheat
894	5R	peroxidase pxdC	stress response	antioxidative and peroxidase activity	72	Q40366_MEDSA
408	5R	thioredoxin superfamily protein	stress response	antioxidative and peroxidase activity	23	A0JQ12_ARATH
623	3R	heat shock 70 kDa protein, mitochondrial	stress response	chaperone activity	22	HSP7M_PEA
305	24R	germin-like protein subfamily 2 member 3	signal transduction	carbohydrate binding	43	GL23_ARATH
457	5R	germin-like protein subfamily 1 member 1	signal transduction	carbohydrate binding	41	GL11_ARATH
543	5R	TCTP(translationally-controlled tumor protein homolog)	signal transduction	calcium ion second messenger	29	TCTP_MEDSA
851	24R	isopentenyl pyrophosphate isomerase	secondary metabolite biosynthesis	terpenoid metabolism	45	Q9AVG8_TOBAC
895	24R	v-type proton ATPase subunit B1	transport	ion transport	29	VATB1_ARATH
			Mtvector_*S. meliloti*			
738	5V	pectinesterase	defense response	cell wall degradation	62	MTR_8g104620
464	5V	RBOH I	defense response	ROS production & hypersensitive response induction	55	RBOHI_ARATH
796	5V	glutathione S-transferase	defense response	ROS detoxification & degradation	53	GSTUM_ARATH
775	3V	PR10-1 protein	defense response	pathogenesis-related protein	52	MTR_2g035100
410	3V	RBOH B	defense response	ROS production & hypersensitive response induction	33	RBOHB_SOLTU
426	5V	peroxidase	stress response	antioxidative and peroxidase activity	84	MTR_4g132110
153	3V	heat shock protein 83	stress response	chaperone activity	73	HSP83_IPONI
923	24V	peroxidase pxdC	stress response	antioxidative and peroxidase activity	68	Q40366_MEDSA
129	3V	chloroplast envelope membrane 70 kDa HSP-related	stress response	ATP binding	45	HSP7E_SPIOL
396	3V	stress responsive alpha-beta barrel domain protein	stress response	uncharacterized protein	35	TC177020
735	3V	nodule-specific cysteine-rich peptide 96	signal transduction	nodulation	75	Q2HW73_MEDTR
417	3V	CBL-interacting protein kinase 15	signal transduction	hormone metabolism	56	CIPKF_ORYSJ
718	5V	auxin responsive SAUR protein	signal transduction	hormone metabolism	47	TC196766
266	3V	dihydroflavanol-4-reductase 1	secondary metabolite biosynthesis	flavanoid biosynthesis	34	Q6TQT1_MEDTR
891	3V	H+-transporting two-sector ATPase, C (AC39) subunit	transport	ion transport	49	MTR_7g009590

MtROP9i_*G. intraradices*						
6	5R	class-10 pathogenesis-related protein 1	defense response	pathogenesis-related protein	6	PR1_MEDSA
10	5R	Pprg2 protein	defense response	pathogenesis-related protein	6	Q8L6K8_MEDSA
52	3R	haem peroxidase, plant/fungal/bacterial	defense response	ROS detoxification & degradation	5	MTR_7g086820
777	3R	MLP-like protein	defense response	pathogenesis-related protein	4	MTR_8g045570
408	5R	endochitinase	defense response	cell wall degradation	3	TC173292
14	3R	peroxidase2	stress response	antioxidative and peroxidase activity	14	O24080_MEDSA
10	3R	heat shock 70 kDa protein, mitochondrial	stress response	chaperone activity	10	HSP7M_PEA
371	5R	prohibitin 1-like protein	stress response	chaperone activity	4	Medtr4g078200.1
707	3R	clathrin assembly protein: At4g32285	stress response	endocytosis	4	CAP1_ARATH
422	5R	inositol-3-phosphate synthase	signal transduction	protein-protein interaction	23	INO1_NICPA
496	24R	GTP-binding protein-related	signal transduction	GTP binding / GTPase activity	13	Q9AY71_ORYSJ
402	24R	soluble inorganic pyrophosphatase	signal transduction	protein-protein interaction	12	Medtr2g010430.1
423	24R	NAD(P)H-dependent 6'-deoxychalcone synthase	secondary metabolite biosynthesis	phytoalexin biosynthesis	24	6DCS_SOYBN
570	24R	Tic22	transport	intracellular transport	14	Medtr3g101630.1
Mtvector_*G. intraradices*						
219	3V	cationic peroxidase 1	defense response	ROS detoxification & degradation	43	PER1_ARAHY
747	24V	pathogenesis-related protein 4A	defense response	pathogenesis-related protein	20	Q9M7D9_PEA
761	5V	glutathione peroxidase	defense response	ROS detoxification & degradation	23	Medtr8g105630.1
570	5V	polygalacturonase inhibitor protein	defense response	cell wall degradation	14	Medtr7g023590.1
862	5V	pathogenesis-related protein 1a	defense response	pathogenesis-related protein	13	MTR_2g012370
262	3V	peroxidase2	stress response	antioxidative and peroxidase activity	23	O24080_MEDSA
22	5V	thioredoxin h1	stress response	antioxidative and peroxidase activity	22	A1BLP6_MEDTR
41	5V	protein P21 kinase inhibitor	stress response	signal transduction inhibitor	17	P21_SOYBN
96	24V	small heat shock protein, chloroplastic	stress response	heat shock protein	10	HS21C_Wheat
772	5V	heat shock protein DnaJ	stress response	chaperone activity	4	MTR_7g114150
477	3V	14-3-3-like protein	signal transduction	protein-protein interaction	34	Medtr3g099380.1
745	5V	caffeic acid 3-O-methyltransferase	signal transduction	protein-protein interaction	14	Medtr4g038440.1
545	3V	blue (Type 1) copper domain	signal transduction	ion binding	12	MTR_7g086090
725	3V	progesterone 5-beta-reductase	secondary metabolite biosynthesis	steroid biosynthesis	16	Medtr3g013890.1
112c	5V	v-H(+)-ATPase subunit A	transport	ion transport	39	D7EYG6_SOYBN

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Curriculum vitae

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Publications and conference contributions

Publications

Kiirika, LM., Bergmann, HF., Schikowsky, C., Wimmer, D., Korte, J., Schmitz, U., Niehaus, K. & Colditz, F. (2012). Silencing of the Rac1 GTPase MtROP9 in *Medicago truncatula* stimulates early mycorrhizal and oomycete root colonizations but negatively affects rhizobial infection. *Plant Physiol*. 159: 1-16.

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Kiirika, LM., Schmitz, U. and Colditz, F. (2013). Proteomic profiling of transgenic *Medicago truncatula* roots defective in ROS signalling after early symbiotic and pathogenic microbial infections (*In preparation*).

Kiirika, LM., Stahl, F. & Wydra., K. (2013). Phenotypic and molecular characterization of resistance induction by single and combined application of chitosan and silicon in tomato against *Ralstonia solanacearum*. *Physiol.* and *Mol. Plant Pathol*. 81: 1-12.

Conference Contributions

Posters
Kiirika, LM., Schmitz, U. and Colditz, F.: Infection proteome of signalling protein MtROP9-interfered *Medicago truncatula* roots and significant changes during ROS defense signalling.
7th European Summer School; "Advanced proteomics", August 2013, Kloster Neustift, Brixen, Italy.

Kiirika, LM., Braun, H-P., Niehaus, K. and Colditz, F.: Functional role of signalling protein MtROP9 during symbiotic and pathogenic interactions in model legume *Medicago truncatula* via molecular and proteomic approaches.
Proteomic Forum, March 2013, Freie Univ. Berlin, Germany.

Kiirika, LM., Korte, J., Schmitz, U., Niehaus, K. and Colditz, F.: Defense proteins during early symbiotic and pathogenic interactions in the model legume *Medicago truncatula*.
TROPENTAG: International Research on Food Security, Natural Resource Management & Rural Development, September 2012, Univ. Göttingen, Germany.

Kiirika, LM., Bergmann, HF., Schikowsky, C., Wimmer, D., Korte, J., Schmitz, U., Niehaus, K. & Colditz, F.: Legume small GTPase MtROP9 and its role in establishment of plant defense during microbial interactions.
58th Plant Protection Conference, September 2012, Technical Universität. Braunschweig, Germany.

Acknowledgements

"Really great people make you feel that you, too, can become great" - Twain Mark

To Prof. Dr. Braun: I express my deepest sense of gratitude for giving me an opportunity to do a PhD in your department. Being an international student, I have never at any one time felt like being in foreign land because of the perfect atmosphere and a nice trained team you spearhead. Furthermore, the profound opportunity you offered me for attending conferences, especially international, provided me with excellent ground for getting not only ideas for scientific progress but also great pleasure to meet new people from different parts of the world. Thank you also for the great care and support you have always accorded me and my family such that I was motivated even when things were not going on well, at times back at home in Kenya.

To Prof. Dr. Schmitz: I equally express my utmost gratitude for the great support you gave me, moreso to ensure that I am able to complete my PhD. I will live to recall your sharply-put and precise advises and corrections during my 3-year period.

To PD. Dr. Colditz: I have no words to express the kind of support you gave me both in and out of the lab. Your friendly supervision approach motivated me to strive to achieve my objectives and gave me confidence always. I can attest to the fact that your training has imparted me know-how, given confidence and equipped me with a solid background ready to hit my career mark. Moreover, the memories of time spent in your 'Medicago Group' while discussing our progress will forever ring at the back of my mind.

Dagmar, Christa and Marianne: Thank you for your continued support in the laboratory in many aspects to ensure every part of my work emerges successfully.

To Holger, Jennifer and the entire AG Braun: I would like to thank you for the wonderful time I have spent with you. I have enjoyed the friendly working environment and always the unforgettable attitude of willingness to help, even when not necessarily being asked. Moreover, the level of security concerns in the group engaged some of us which comes out really as so much fun. Thank you also for standing with me during my worst moment in life of bereavement.

In my office shared with **Peter**: You made my environment so perfect that I will miss for the rest of my life if not eons of my existence. Thank you especially for being a nice friend and supporting me always on all sorts of problems. Off course I cannot forget our greatest fun of playing soccer together. To our FC Coomassie team: kudos! for wining 2-times medal in a row, during my era.

To all colleagues and friends especially from **AG C. Peterhänsel group** who supported me in many ways and all others not mentioned here, thank you.

My lovely Mum Sarah and my Dad Erastus: Although you both painfully departed us during my study period in a foreign land, your everlasting love and bringing up will forever shape my destiny! You worked hard to make me what I am today and without you I would not exist. Truly, God knows why you were to leave us and no to pocket the pride of your son, but I am sure of meeting you one day and posting you of my achievements! **My Grandma Julia:** You are the world's best grandma I could wish for.

Overall, I thank God for the awesome love and grace that I have seen and continue to see daily!!

Declaration

I hereby declare that this dissertation entitled “Molecular aspects of early symbiotic and pathogenic infection in *Medicago truncatula* Gaertn” submitted for the Degree of Doctor of Philosophy is my original work and the materials/resources and figures used from other institutions for assisting in explanations are fully specified.

The thesis has not been submitted to any other University or Institution for the award of any degree, diploma, associateship, fellowship or similar other titles

Hannover, Date: 14th November 2013