Physiological and molecular basis of *Azospirillum-Arabidopsis* Interaction

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Dedicated to

My father

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Abbreviations

ABA	Abscisic Acid
ACC	Aminocyclopropane-1-Carboxylic acid
Acds	ACC deaminase
AGI	Arabidopsis Gene Initiative
AM	Arbuscular Mycorrhizae
BHLH	Basic Helix Loop Helix
BLAST	Basic Local Alignment Search Tool
BR	Brassinosteroid
BSA	Bovine Serum Albumin
CFU	Colony Forming Units
cDNA	Complementary DNA
Col-0	Columbia 0
CPS1	Counts Per Second 1
DAMP	Damage Associated Molecular Pattern
DEPC	Diethylpyrocarbonat
DGPP	Diacylglycerol Pyrophosphate
DNA	Deoxyribonucleic Acid
dNTP	deoxynucleoside Triphosphate
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
ECM	Ectomycorrhizae
EDTA	Ethylendiamintetraacetat
EF TU	Elongation Factor TU
EPS	Exopolysaccharides
ER	Endoplasmatisches Reticulum
EST	Expressed Sequence Tag
ETI	Effectors-Triggered Immunity
ETS	Effectors-Triggered Susceptibility
Flg	Flagelline
GA	Gibberelic Acid
GFP	Green Fluorescence Protein
GltS	Glutamate Synthase
GO	Gene Ontology
GS	Glutamine Synthetase

Abbreviations

GSLs	Glucosinolates
HATS	High Affinity Transport System
HPLC	High Performance Liquid Chromatography
hpi	Hours post inoculation
HR	Hypersensitive Response
HRP	Horse Reddish Peroxidases
IAA	Indole Acetic Acid
IaaH	Indole-3-acetaldehyde Hydrolase
IAN	Indole-3-Acetonitrile
IAOx	Indole-3-Acetaldoxime
IpyA	Indole-3-pyruvic Acid
Ipdc	Indole-3-pyruvic acid decarboxylase
ISR	Induced Systemic Resistance
KDa	Kilodalton
LFC	Log Fold Change
LP	Lipo Peptide
LSM	Laser Scanning Microscope
LysM	Lysine Motif
MAMPs	Microbe Associated Molecular Patterns
Met	Methionine
MOMP	Major Outer Membrane Protein
mRNA	messenger RNA
MS	Murashige & Skoog
NA	Not Available
NB	Nucleotide Binding
NFS	Nitrogen Fixing Symbioses
NO	Nitric Oxide
PBS	Phosphate Buffered Saline
PC	Phosphatidyl Choline
PCD	Programmed Cell Death
PCR	Polymerase Chain Reaction
PE	Phosphatidyl Ethanolamine
PEG	Polyethylenglycol

PEG	Polyethylenglycol
PGPB	Plant Growth Promoting Bacteria
PGPR	Plant Growth Promoting Rhizobacteria
PHB	Poly-b-Hydroxy Butyrate
Pi	Inorganic phosphate
PLC	Phospholipase C
PLD	Phospholipase D
PRRs	Pattern Recognition Receptors
PTI	PAMPs-Triggered Immunity
Pv	Pathovar
qRT-PCR	Quantitative Real Time PCR
RLKs	Receptor Like Kinases
RNA	Ribonucleic Acid
RNase	Ribonuclease
ROS	Reactive Oxygen Species
RPM	Rotations Per Minute
RT	Reverse transcriptase / Room temprature
SAR	Systemic acquired resistance
Sbp A	Sugar binding protein A
Ser/thr	Serine/ threonine
SDS-PAGE	Sodium Dodecylsulfate -Polyacrylamide Gel Electrophoresis
TAM	Tryptamine
TBE	Tris-Borat-EDTA-Buffer
TE	Tris-EDTA-Buffer
TF	Transcription factor
TENS	TE-NaOH-SDS-Buffer
Trp	Tryptophan
USP	Universal Stress Protein
WGA	Wheat Germ Agglutinin
WS	Wassilewskija

Summary

The present study was aimed at revealing the early signalling events during the interaction of the diazotrophic soil bacterium *Azospirillum brasilense* with its host plant *Arabidopsis thaliana*. Furthermore, taking advantage of the micro array technique, a comprehensive overview of *Arabidopsis* genes has been undertaken which are affected upon association with *A. brasilense*

The characterization of the early responses of *Arabidopsis* plants upon inoculation with *Azospirillum brasilense* strain Sp7 clearly indicated parallels with the initial events in plant pathogen interaction. For instance, not only bacterial preprations (lysates) form *Azospirillum* elicited an apoplastic alkalinization of the culture medium, but also the live bacteria, which were even more effective. Besides, in a luminol based assay, the bacterial lysates triggered production of the reactive oxygen species (ROS) in the *Arabidopsis* leaf discs. Interestingly, the elongation factor receptor mutants (*efr*) were completely insensitive to *Azospirillum*, suggesting elongation factor Tu (EF-TU) recognition as elicitor by *Arabidopsis*. This hypothesis was further validated with a bioinformatic approach. The N terminus initial 26 amino acids from *Azospirillum* EF-TU gene (elf26) showed more similarity to the elf26 sequences of bacteria like *Agrobacterium tumefaciens* which elicit responses in the plants through EF-TU rather than *Pseudomonas syringae* where the potent elicitor is flagellin 22.

Universal transcriptome profiling of *Arabidopsis thaliana* seedlings upon inoculation with *Azospirillum brasilense* over a time course of six, twenty four and ninty six hours revealed very little genetic responses in the early time points. However, a bulk of genes was differentially regulated in 96 hours post inoculation (96hpi). The nature of these genes indicated that the bacterial treatment, among others, greatly affect the processes like cell wall modification, hormone metabolism, stress and secondary metabolism. Additionally expression levels of a numer of transcription factors (TFs) related to basic helix loop helix (BHLH) and MYB domain containing TF families were altered with *Azospirillum* inoculation. Particularly the BHLH TFs were among the most highly regulated genes.

The array results from *Azospirillum* treated plants were further compared with the already available data emnating from treatment with flagellin 22 (flg22), oligogalacturonides (OGs) and *Agrobacterium tumefaciens.* Noteworthy, very different set of genes were affected upon inoculation with *Azospirillum* in relation to other treatments. Secondly a cluster of proteins involved in the biosynthesis of aliphatic glucosinolates (GSL) were uniquely induced upon Sp7 exposure. Genes operating in flavonoid biosynthesis also showed a distinct regulation trend in the comparative analysis.

Taken together, the study in question provides insights into the early signalling events in the context of *Azospirillum-Arabidopsis* association and the bacterial signals recognized by the plants. The array data, at the same time, elucidates the genetic factors of *Arabidopsis* triggered upon association with *Azospirillum brasilense*.

Zusammenfassung

Die vorliegende Arbeit befasst sich mit den physiologischen und genetischen Reaktionen im Zuge der Interaktion von *Arabidopsis thaliana* mit dem freilebenden, Stickstoff-fixierenden Bodenbakterium *Azospirillum brasilense*.

Qualitativ konnten gemeinsame Mechanismen der frühen physiologischen Antworten von Arabidopsis auf Lysate von mutualistischen (*Azospirillum brasilense)* oder pathogenen (*Pseudomonas syringae* und *Agrobacterium tumefaciens*) Mikroorganismen festgestellt werden. So reagierten Arabidopsis (Col-0) Pflanzen auf Lysate dieser Bakterien mit einem Anstieg der cytosolischen Calcium-Konzentration sowie des extrazellulären pH Werts, mit der Bildung reaktiver Sauerstoffspezies und einer Depolarisierung des Membranpotentials. Diese Antworten untschieden sich jedoch zum Teil erheblich in ihrer Amplitude. Weitere Untersuchungen konnten zeigen, dass Flagellenproteine von Azospirillum nicht durch *Arabidopsis* erkannt werden. Somit unterscheidet sich der Erkennungsmechanismus der Azospirillen von dem der Pseudomonaden, welche aufgrund ihrer Flagellenproteine durch den *FLAGELLINSENSING-2 (FLS2)* Rezeptor in *Arabidopsis* perzipiert werden. Die *Arabidopsis* Mutante *ELONGATIONFACTOR RECEPTOR (efr)* war insensitiv gegenüber Azospirillumlysaten. Dies legte nahe, dass die Erkennung von *Azosprillum* über eine Erkennung des bakteriellen Elongationsfaktors (EF-Tu) durch den EFR Rezeptor verläuft. Die anschließende Klonierung des *Azospirillum* EF-Tu Gens zeigte positionspezifische Unterschiede in der abgeleiteten Aminosäuresequenz gegenüber Referenzsequenzen aus *Escherichia coli* oder *Agrobacterium tumefaciens* und erklärt somit die „imperfekte“ Erkennung durch den EFR Rezeptor.

Der zeitliche Verlauf der genetischen Antwort von Arabidopsis im Zuge der Interaktion mit *Azospirillum* wurde mit Hilfe „Micro-Array“ basierter Transkriptionansanalysen 6, 24 und 96 Stunden nach Inokulation (hpi) der Pflanzen untersucht. Dabei wurden nach 6 und 24 hpi lediglich 30 bzw. 60 differenziell regulierte Transkripte gefunden. Diese Beobachtung steht im Gegensatz

zu Studien pathogener Elizitoren wie Flagellinen, in welchen bereits nach wenigen Stunden mehr als eintausend differenziell regulierte Transkripte in *Arabidopsis* gefunden wurden. Dieser Effekt konnte in den Interaktionsstudien mit *Azospirillum* erst nach 96 hpi beobachtet werden. Die Analyse der genetischen Antwort ergab, dass 96 hpi insbesondere Gene in ihrer Expression verändert waren, deren Produkte im Zusammenhang mit Zellwandmodifikationen, dem Hormonmetabolismus, der Stressanpassung sowie der sekundären Metabolismus stehen. Darüber hinaus konnten Gene aus der Familie der sog. „basic-helix-loop-helix“ und „MYB“ Transkriptionsfaktoren identifiziert werden, die einer spezifischen Regulation durch Azospirillum unterlagen. Die vergleichende Analyse der Araydaten mit Datensätzen, die im Zuge von Pathogen-*Arabidopsis* Interaktionen gewonnen wurden zeigte, dass insbesondere die Biosynthese von aliphatischen Glykosiden und Flavonolen eine typische Antwort der Pflanze auf die mutualistischen *Azospirillum* Bakterien darstellt.

Die vorgestellte Arbeit liefert somit erste Erkenntnisse zur physiologischen und genetischen Antwort von *Arabidopsis* auf *Azospirillum* und ermöglicht die vergleichende Betrachtung dieser Antworten im Kontext der Interaktion von Pflanzen mit pathogenen Mikroorganismen. Die im Rahmen dieser Arbeit identifizierten, differenziell regulierten Gene bieten neue Ansatzpunkte zum vertieften Studium der Wechselwirkung von mutualistischen, wachstumsfördernden Bakterien mit höheren Pflanzen.

1 Introduction

1.1 Plants and the rhizospheric microbes

Plants do not live in isolation. Rather they are in constant interactions particularly with different microorganisms in the rhizosphere. These associations are traditionally categorized as: (i) negative (pathogenic) interactions; (ii) positive (symbiotic or associative) interactions and (iii) neutral interactions (Singh et al., 2004).

The term pathogenesis is usually applied to designate the negative nature of the interaction between the interacting organisms. In the gene ontology (GO) terms, pathogenesis has been defined as “The set of specific processes that generate the ability of an organism to cause disease in another”(http://www.geneontology.org). The process usually includes the multiplication of the microbes such as bacterium, fungus, oomycete, nematode or protozoan in a host. The level of such proliferation to become lethal depends on many factors especially the context of the interaction. For instance, the hypersensitive response (HR) (a mechanism used by the plants to restrict the spread of pathogenic bacteria through its resistance (R) gene products) is whether detrimental or beneficial to the plant, depends on the necrotrophic or bio (hemi) trophic nature of the invading microbe.

Harmful microbes could bring different deleterious effects to the growth and development of plants mainly through production of metabolites like phytotoxins or hormones, competing for nutrients and limiting or inhibiting the beneficial impacts of other micro organisms (Nehl et al., 1996).

The second category of relationship in the plant microbe perspective is neutral interactions. As the name itself indicates, the neutral interaction of the rhizosphere probably has no effect on both partners, however another term used in the context of plant microbe interaction is commensalism. This describes the unidirectional relationship between the two populations, where one benefits and the other one is unaffected.

Unlike the antagonistic interaction, where pathogenic microbes cause damage, often resulting in retarded plant growth and reproduction, the mutualism brings about benefits to the interacting partners. Mycorrhizal and diazotrophic associations with plants explain the term mutualism in a candid way.

1.2 Mycorrhizal interactions

Mycorrhizae generally represent the many diverse mutualistic relationship of the fungus with the host plant where the fungus colonize the root of the plant either intra- or extracellularly. Depending on their colonization pattern of plant roots, mycorrhizae are termed ectomycorrhizae (ECM), when their hyphae remain extracellular and endomycorrhizas when the hyphae pierce the root cells to establish an intracellular symbiosis. The arbuscular mycorrhizae (AM) fall in the latter category along with restricted ericoid and orchid mycorrhizae. While the trees and shrubs generally represent the ectomycorrhizae.

Colonization is initiated when the hyphae contact the plant root. The steps, however, involved in colonization vary with the type of mycorrhiza involved (Smith and Read, 2008). In the following lines, arbuscular mycorrhizae will primarily be focused as representative of the mycorrizal interactions by virtue of being the most widespread symbiosis among different plant taxa (Bonfante and Genre, 2008).

Arbuscular mycorrhizae (AM) are observed as resting spores in the absence of an intimate association with the plant in the soil. Upon germination, the hyphal germ tube looks for host roots through the soil. Upon finding a host, the fungus forms an appressorium on the root surface which helps the fungus entering the root. After entering the inner cortex of the root cell, they form arbuscules, which are dichotomously branched hyphae. These structures are thought to be channels of nutrient exchange between the partners (Harrison, 2005).

Both AM and the plants are suggested to perceive signals released by the respective partner prior to their physical intimacy. Arbuscular mycorrhizae fungi

spores, for instance, can germinate in water. The germination in water is stimulated by plant root exudates and volatiles, including CO_2, indicating a

sensory system for components of the rhizosphere (B´ecard and Pich´e, 1989; Gianinazzi-Pearson V et al., 1989). Similarly enhanced growth and hyphal branching were observed even when the hyphae reach the vicinity of a root of the host plant, signifying the perception of root exudate signals by AM fungi (Buee et al., 2000; Giovannetti M et al., 1993). It has now been demonstrated that hyphal branching is induced by plant secreted signals strigolactones (Akiyama et al., 2005), which are comparable to flavonoids in legume- *Rhizobia* interaction. However, perception of strigolactones by the fungus is yet to be conclusively elucidated.

As far as receiving fungal signals by plants is concerned, Nod factor like signals (Myc factors) were suggested for the AM fungi, but direct evidence is lacking. However work with *Medicago truncatula* indicated the presence of a specific AM fungal signal molecule. The authors observed induction of a *MtENOD11*gene (Chabaud M et al., 2002; Journet et al., 2001) prior to direct contact with the AM fungi (Kosuta et al., 2003). The gene induction response was found specific for various AM fungal species but not for pathogenic fungi.

The headway in understanding the intracellular infection and signaling mechanisms between Mycorrhiza and plant have been facilitated by developments in recognition of common symbiosis (Sym) pathways in nitrogen fixing symbioses (NFS) between legumes and *Rhizobia* and in legume arbuscular *Mycorrhiza* interaction. Subsequent analysis revealed the existence of at least partially similar genetic basis for nodulation and AM development, signifying an evolution of the NFS pathway from the more ancient AM pathway (Gianinazzi-Pearson and Denarie, 1997; van Rhijn et al., 1997).

A number of SYM genes implicated in the early AM signaling operate upstream of a channel protein (DMI1/POLLUX/CASTOR) and a calcium-calmodulin protein kinase (DMI3, *Ps*SYM19 and *Ps*SYM30) (Harrison, 2005; Kistner et al., 2005). In NFS, these genes function in response to Nod factors and are involved in changes of the Calcium status in root hairs. These results indicate a

putative action of calcium as a crucial second messenger during the signaling pathway in the early AM symbiosis (Levy et al., 2004; Oldroyd et al., 2005).

Some advances in respect of the molecular mechanism underlying nutrient exchange have been made with the advent of new techniques. AM, for instance, through their phosphate transporters take up inorganic phosphate (Pi) from the soil and ensure its availability to plants (Harrison and van Buuren, 1995). The plant mycorrhiza specific phosphate transporters further enable phosphate supply in plant cells. Interestingly these plant transporters have also been linked to the arbuscule vitality and sustain development of the fungus (Javot et al., 2007).

Genes implicated in organic and inorganic uptake of Nitrogen have been identified in both AM and ectomycorrhizal (ECM) fungi (Cappellazzo et al., 2008; Lucic et al., 2008). When mycorrhization takes place, plant Nitrogen transporters are activated (Guether et al., 2009a; Guether et al., 2009b) suggesting N release from the fungus to the plant.

The mycorrhizal fungi which rely for organic Carbon (C) supply on its photosynthetic partner, utilize the photosynthate both for production of vegetative and reproductive structures and in respiration to support growth and maintenance, including nutrient uptake. The transfer of Carbon is mostly directed from the plant towards the mycorrhizal fungus. However the flow in the reverse direction is also observed in few cases. For example, in orchid mycorrhizae or in other heterotrophic plants, Carbon moves from the fungus toward the plant (Selosse and Roy, 2009). Whatever the case is, the genes participating in the C transfer are not well understood. Few representative exceptions in this regard are the glomeromycotan G*eosiphon pyriforme* gene (Schussler et al., 2006), interacting with a cyanobacterium, and *Aspergillus niger (Am*Mst1) gene from the ECM fungus *Amanita muscaria* (Nehls et al., 1998).

It has been demonstrated that in AM interacting plants the efficiency of uptake of elements like Zn, Cu and Mn is elevated. Parallel to these reports, there also exists a role of AM colonization in decreasing Zn accumulation and resultantly

low plant toxicity in soils with high Zn contents (Burleigh et al., 2003; Li and Christie, 2001; Zhu et al., 2001).

AM fungi are relatively better investigated with reference to the effect of mycorrhizal colonization on plant defenses. The interaction of the AM brings about minor, transitory defense responses, which are followed by general suppression. When compared to the plant responses to pathogens, we see no major changes in synthesis of lignin or callose in plant cells associated with AM. Furthermore, transcriptional activation of genes in the phenylpropanoid pathway like phenylalanine ammonia lyase (PAL), chalcone synthase (CHS) and isoflavone reductase (IFR) is noted in *Medicago* and *Phaseolus*. The induction of chalcone isomerase (CHI) was however limited to *Medicago* only. These defense responses, the extent of which in most cases is weaker as compared to the plant responses to pathogen, are thought to confer some pre-immunity on the plants, minimizing thus the effects of subsequent attack by damaging pathogens (Cordier et al., 1998).

1.3 Diazotrophs

A lion’s share of the nitrogen fixation process is performed by microbes (prokaryotes). These microorganisms, by virtue of their ability to fix atmospheric nitrogen to ammonia using the enzyme nitrogenase, are called diazotrophs. By convention, three classes of diazotrophs are usually described, i) non-symbiotic or free living; ii) associative symbiotic and iii) symbiotic diazotrophs. The free living diazotrophs thrive without associating themselves to the plant roots. *Azotobacter* is a classic example of such microbes. Associative symbionts, like *Azospirillum*, on the other hand, are found partly within the root and partly outside the plant without inducing nodule formation in the plants. The last category represented by the gram-positive filamentous actinomycete (*Frankia*) (Benson and Silvester, 1993) and the gram-negative proteobacteria (*Rhizobia)* are not only capable of infecting the plant but also induce nodule-like structures on plant roots.

1.3.1 Rhizobia-legume mutualism

The nodulating plants interacting with *Rhizobia* belong to two different families, namely *Leguminosae* (subfamilies *Caesalpinioideae*, *Mimosoideae* and *Papilionoideae*) and *Parasponia* genus from the *Ulmaceae* family (Sprent, 2007).

Rhizobia are well known for their ability to form mutualistic endosymbioses with legumes. The interaction results in the formation of root nodules, which house nitrogen fixation. These nodules are polyploid cells (Kondorosi et al., 2000) containing a large number of bacteroids inside. Bacteroids are basically differentiated *Rhizobia,* supplying plants with ammonium after reducing atmospheric nitrogen (Prell and Poole, 2006).

Colonization of the root surface by *Rhizobia* is accompanied by morphological changes in the epidermis and in the induction of gene expression. The early nodulin genes ENOD12 and ENOD11, which encode proline-rich proteins, are the best and well investigated examples in this regard (Journet et al., 2001; Scheres et al., 1990). The changes in the root morphology give root hairs a curling shape which is followed by formation of an infection thread in epidermal cells. The nodule primordium in the inner cortex cells is formed after a limited number of cell divisions. The infection thread loaded with bacteria is then directed towards the nodule primordium, where it releases the bacteria. These *Rhizobia* subsequently start N fixation once they are differentiated into their symbiotic form. The role of bacterial secreted signaling molecules, called Nod factors, is of prime importance in these early events (Geurts and Bisseling, 2002).

In a compatible interaction, initiation of nodulation is observed when most *Rhizobia* secrete lipochitooligosaccharidic nodulation factors (Nod factors), in response to plant flavonoid signals. These plant signals activate the bacterial transcriptional regulator NodD that in turn induces the transcription of other bacterial nodulation genes involved in the synthesis of Nod factors. After being recognized by the plants, these factors ensure the entry of the bacterium into

the legume root. Beside Nod factors *Rhizobia* also use additional signals like secreted proteins or surface polysaccharides in the process of interacting with legumes.

One of the earliest signaling events related to the perception and recognition of Nod factors in the plant root cells is plasma membrane depolarization, observed after a lag period of 1 minute after Nod factor addition (Ehrhardt et al., 1992). This depolarization is linked to a rapid calcium influx observed within seconds of the Nod factor addition followed by a chloride efflux (Felle et al., 1999) . In addition to the increase of the intracellular Calcium levels in plant root hairs, strong cytosolic Calcium oscillations have also been reported (Oldroyd and Downie, 2004). Similarly a rapid apoplastic alkalinization of 0.2-0.3 pH units also featured the initial responses of the plant to Nod factors (Felle et al., 1996).

The effect of the Nod factors on phospholipid signaling is observed by an increase in the concentrations of phosphatidic acid (PA) and diacylglycerol pyrophosphate (DGPP) through the activation of both phospholipase C (PLC) and phospholipase D (PLD). Notably the activation of these both phospholipases is considered to be essential for the induction of root hair deformation (den Hartog et al., 2001).

In addition to the said responses, an induction of plant genes in response to Nod factors has also been observed. Some of these genes encode lysine motif (LysM) Ser/Thr receptor kinases and are predicted to be Nod factor receptors (Madsen et al., 2003). These receptors function upstream of ion channels encoding genes putatively involved in intracellular Calcium fluctuations, imperative for the nodulation process (Imaizumi-Anraku et al., 2005). The regulation of several transcription factors is observed to take place downstream of the ion channel proteins. An example in this context is the *Lotus japonica* gene LjNIN which encodes a bZIP transcription factor (Schauser et al., 1999).et al, 1999). A battery of additional genes like DMI1 & DMI2 (does not make infections) have been found operating downstream of LysM, mutants of which

are unable to induce root hair deformation and rapid Ca^{2+}-influx in response to Nod factors (Gibson et al., 2008) and the references contained therein.

Nod factors have also been shown to affect the levels of reactive oxygen species (ROS) in the plant. The initial stages of symbiosis are characterized by reduced ROS production favoring early morphological responses like root hair deformation and curling (Lohar et al., 2007). However, contrasting reports also exist showing changes in the ROS levels in the living root hair cells of *Phaseolus vulgaris* seconds after addition of NFs (Cardenas et al., 2008). These altered ROS status was accompanied by increased cytosolic Calcium levels. Elevated ROS levels are also recorded after rhizobial infection in developed nodules (Rubio et al., 2004) and during infection thread formation (Ramu et al., 2002; Santos et al., 2001). These observations along with a number of others clearly emphasize the role of ROS in legume-rhizobia interaction not only at the initial stages of interaction but also to later events like infection thread formation.

1.3.2 Plant growth promoting Rhizobacteria / associative symbionts

Bacteria are by far the most abundant microorganisms occurring among the plethora of different types found in the soil. In addition to bacteria which were found to be bound on the surface of soil particles, many more of them interact with the roots of plants in the rhizosphere. A portion of these rhizospheric bacteria are capable of exerting growth stimulatory effects on plants and are referred to as plant growth promoting rhizobacteria PGPR. Irrespective of the epiphytic or endophytic nature of the PGPR, their infection is characterized by the absence of any phenotypic structures like nodules on the host plant. Some *Rhizobia* which are interacting with non-legumes can also behave in a PGPR manner (Sessitsch et al., 2002).
The mechanisms by which PGPR stimulate plant growth can broadly be categorized as direct or indirect (Glick, 1995). Featured direct mechanisms include enhanced plant growth through production of plant hormones such as indole acetic acid (IAA), gibberelic acid (GA) and cytokinins, eliciting plant

volatile production, lowering ethylene levels in plants, induced systemic resistance and ensuring nutrient availability to plants through phosphate solubillization (Glick, 1995; Idriss et al., 2002) and the conversion of molecular nitrogen into ammonia by virtue of the nitrogenase enzyme complex and by inducing NH_4^{+-}transporters (Becker et al., 2002; Steenhoudt and Vanderleyden, 2000).

Concerning indirect mechanisms, PGPR promote plant growth by attenuating harmful effects of phytopathogens Similarly they may also stimulate other beneficial symbioses or degrade xenobiotics in the contaminated soil (Jacobsen, 1997).

Based on the capability of the PGPR to make more nutrients available to plants, stimulate the plant growth by different determinants, especially phytohormones, degrade organic pollutants and to produce antimicrobial compounds, they have also been classified as biofertilizers, phytostimulators, rhizoremediators and bio pesticides (Somers et al., 2004).

The initial studies on PGPR were limited to few species, but with the passage of time the spectrum has widened to a great extent. To date, it covers a range of bacterial taxa (Lucy et al., 2004) like *Bacillus* and *Pseudomonas*. Many species of these genera are now well known for their growth promoting effects.

Free-living diazotrophs which are able to fix nitrogen constitute one of the most important classes of plant growth promoting Rhizobacteria. Various bacteria which employ, among others, the above mentioned way to stimulate plant growth include *Gluconacetobacter*, *Acetobacter diazotrophicus*, *Herbaspirillum seropedicae*, *Burkholderia spp.*, *Azoarcus spp.* and *Azotobacter*. *Azospirillum* is one of the better investigated among these genera.

1.3.3 *Azospirillum*

Historically speaking, Beijerinck in 1922 first observed the growth of a spirillum-like bacterium in Nitrogen deficient malate- or lactate-based media. He initially considered the organism as a member of the genus *Spirillum* and a bridging link

between the genus *Spirillum* and the genus *Azotobacter*. The organism named "*Azotobacter spirillum*" was later renamed as "*Spirillum lipoferum*" (Beijerinck, 1925) Subsequent studies failed to show N_2 fixation by pure culture, and the organism did not came into limelight for many years, except for a few scattered reports. It was in 1963, that Becking (and coworkers) were able to isolate an organism resembling *S. lipoferum* that undoubtedly exhibited nitrogenase activity (Becking, 1963). However, the rediscovery of the bacteria in 1970s is attributed to Johanna Döbereiner and her collaborators (Döbereiner and Day, 1976). Because of the similarity to Beijerinck's description of *S. lipoferum*, especially with regard to growth on glucose or mannitol media and formation of *spirillum*-shaped cells under certain conditions, they were named *Azospirillum lipoferum* (Tarrand et al., 1978). Since then many species have been described, however, *Azospirillum brasilense* and *A. lipoferum* are still considered as the two most investigated species of this genus. The complete list of the *Azospirillum* species isolated to date is mentioned in table 1.

Azospirillum, a free living PGPB has repeatedly been isolated from the rhizosphere of many grasses, cereals and other crops all over the world both from temperate and tropical climate (Steenhoudt and Vanderleyden, 2000).. Bacteria of this genus *Azospirillum* are gram negative and motile having a single polar flagellum. In addition to that several lateral flagella of shorter lengths, are observed in *A. lipoferum* and *A. brasilense* on soft nutrient agar, where swarming is experienced (Hall and Krieg, 1983).

	Species	References
1	*Azospirillum lipoferum*	(Beijerinck, 1925; Tarrand et al., 1978)
2	*Azospirillum brasilense*	(Tarrand et al., 1978)
	Azospirillum amazonense	(Magalhles et al., 1983)
4	*Azospirillum halopraeferens*	(Reinhold et al., 1987)
5	*Azospirillum irakense*	(Khammas et al., 1989)
6	*Azospirillum largimobile*	(Sly and Stackebrandt, 1999)
7	*Azospirillum doebereinerae*	(Eckert et al., 2001)
8	*Azospirillum oryzae*	(Xie and Yokota, 2005)
9	*Azospirillum melinis*	(Peng et al., 2006)
10	*Azospirillum canadense*	(Mehnaz et al., 2007a)
11	*Azospirillum zeae*	(Mehnaz et al., 2007b)
12	*Azospirillum rugosum*	(Young et al., 2008)
13	*Azospirillum picis*	(Lin et al., 2009)
14	*Azospirillum palatum*	(Zhou et al., 2009)
15	*Azospirillum thiophilum*	(Lavrinenko et al., 2010)

Table 1 List of the *Azospirillum* species isolated up till now

In order to survive in the competitive rhizosphere, *Azospirilla* resort to various strategies including production of some antibiotics and binding iron. The binding of iron, which is carried out through siderophores, results in limiting the availability of that essential element to the competing rhizospheric population. Siderophores are low molecular-weight molecules which have the ability to bind iron. Ferrus (Fe) limiting conditions are best suited for the production of siderophores. The same Fe-binding system also prevails for different *Azospirillum* species including *A.brasilense* (Bachhawat and Ghosh, 1987) and *A.lipoferum* (Saxena et al., 1986).

Scattered information was available about the genome of various *Azospirillum* species. Very recently the complete genome sequence for one of the endophytic *Azospirillum* sp. strain B510 has been released (Kaneko et al.,

2010). The genome consists of a single chromosome and six plasmids. The number of potential protein coding genes has been described as 2893. The sequence also highlighted the identification of three putative plant hormone-related genes encoding tryptophan 2-monooxytenase (iaaM) and indole-3-acetaldehyde hydrolase (iaaH), which are involved in IAA biosynthesis, and ACC deaminase (acdS), which reduces ethylene levels. However no ipdC homologue was found in the B510 genome.

1.3.3.1 Interaction with plants

The growth promoting effects of *Azospirillum,* which were once considered specific to cereals, are now known for a wide variety of hosts including crop plants (Bashan et al., 2004), trees, desert plants like M*esquite amargo* (Leyva and Bashan, 2008) and even microalgae (Gonzalez and Bashan, 2000).

Azospirilla is generally a rhizosphere bacterium, but displays strain-specific differences in the way and to the extent they colonize roots. They predominantly colonize the root surface and only a few strains are able to infect plants (Patriquin et al., 1983). The bacterial entry into the roots is via lysed root hairs and void spaces created by epithelial desquamation and lateral root emergence (Umali-Garcia et al., 1980). *Azospirilla* have been found on the surface of roots, in the outer and inner cortex as well as in the stele of field-grown maize (Dobereiner and Baldani, 1979; Patriquin and Dobereiner, 1978).

These results pertaining to the localization of bacteria were then based on the old techniques like non- specific staining. However with the advancements in the related techniques these observations have now been validated with in-situ hybridization and comparatively new approaches like specific fluorescent oligonucleotide probes and confocal laser scanning microscopy (Assmus et al., 1995) , strain-specific monoclonal antibodies (Schloter and Hartmann, 1998), or nifH-gus fusions (Van de Broek et al., 1993) specially for the strains Sp7 and Sp245, which are respectively found inside and outside the root cell.

On the basis of experiments involving inoculation with a gusA-labelled derivative of strain Sp245 it has been shown that initial accumulation of bacteria

occurs in the root-hair zone in depressions between epidermal cells and at sites of lateral root emergence. Its spread to the other parts of the root is, however, dependent on the status of the nitrogen and carbon sources present in solution (Van de Broek et al., 1993).

Significant colonization serves as a prerequisite to get the desired responses from *Azospirillum* inoculation. In case the bacteria are not attached to root epidermal cells, the excreted substances by the bacteria are at the mercy of nutritionally versatile microorganisms in the rhizosphere before reaching the plant. No or very little positive effects thus result from inappropriate colonization (Benizri et al., 2001; Hecht-Buchholz, 1998).The proper attachment, in this perspective, attains a great importance.

The mechanism that *Azospirillum* adopts to attach to roots is a two step process called adsorption and anchoring. As far as adsorption phase is concerned, it is shorter, weaker and more rapid than the anchoring phase. Moreover the adsorption is reversible while the anchoring is irreversible.

The adsorption phase is said to be affected not only by the bacterial concentration and duration of its contact with the plant but possibly also by the bacterial cell surface lectins. Its involvement, therefore, in colonization and recognition of root surfaces cannot be overruled (Castellanos et al., 1998). A major outer membrane protein (MOMP), of about 42 kDa identified in *A. brasilense*, has been proposed to interact with exopolysaccharides (EPS) under certain growth conditions leading to aggregation and flocculation (Burdman et al., 1999). The protein showed greater binding affinity towards root extracts of cereals than of tomato and legumes. Additionally, in an in vitro adhesion assay it also showed the same affinity towards different cereal seedlings (Burdman et al., 2001). It was also observed that surface adsorption, depending on the strain, was found saturated between 3-24 hours. However, earlier root treatment with N-acetyl-D-glucosamine partially inhibited the attachment of bacteria to the roots (Yegorenkova et al., 2001). These findings possibly point towards an involvement of lectins in the attachment process.

Lectins are proteins that are capable of recognizing and reversibly binding specific sugar chains of glycosylated molecules (Goldstein et al., 1980). Among the array of lectins, wheat germ agglutinin (WGA) (LeVine et al., 1972) is one of the best grass studied lectin. WGA is found on the entire surface of root seedlings and on the root tips of adult wheat plants and can thus putatively contribute to bacterial adhesion to the root surface, leading to wheat-root colonization. Different *Azospirillum* species have been shown to bind to WGA indicating the presence of specific sugar bearing receptors on the cell surface (Del Gallo et al., 1989) , although the molecular genetics still needs to be elucidated.

On the other hand root lectins such as wheat germ agglutinin (WGA) are suggested as a signal molecule in the *Azospirillum* /wheat roots interaction. Available reports indicate an induction of changes in the cellular metabolism of *A. brasilense* Sp245 and promoted N_2 fixation, excretion of NH_4^+ ions and indole-3-acetic acid (IAA) biosynthesis upon binding of WGA to the roots (Antonyuk et al., 1995; Antonyuk et al., 1993). The same authors in their later reports further elaborated the issue by pinpointing modifications of the phospholipid fraction of *A. brasilense* membranes by WGA supposedly bringing about transmembrane signaling and cell response to WGA (Antonyuk et al., 1999). Similarly, WGA binding resulted in enhanced N-fixation and higher expression of *Azospirillum* nif genes (Karpati et al., 1999). Although WGA binding to *Azospirillum* has been defined, the components of the cell surface of *A. brasilense* responsible for binding of WGA still await elucidation.

In addition to lectins and EPS fractions, still there are other players involved in bacterium plant interaction. The reserve material poly-b-hydroxybutyrate (PHB) is one of them.

The plant root exudates enable the *Azospirillum* to colonize plant root surfaces. In the bacterial perspective, a role of chemotaxis and motility related genes responding to these exudates look to play a key role in the colonization process. This view is seconded by studies utilizing non flagellated mutants and mutants with compromised chemotactic motility towards various amino acids, sugars,

and organic acids. The extent of colonization was much lower in these mutants as compared to wild type bacteria (Vande Broek et al., 1998). Similarly, data on some other mutants like *A. brasilense* sugar-binding protein A (*SbpA*) involved in chemotaxis towards different sugars and *flcA* with reduced swarming, wheat root colonization, adhesion potential etc. clearly demonstrated the imperative nature of chemotaxis and bacterial motility in root colonization. Interestingly, a strong induction of SbpA was observed in *A. brasilense* upon addition of root exudates of plants to the bacterial growth medium (Van Bastelaere et al., 1999). The SbpA protein is thought to be involved in the uptake of D-galactose by a protein-binding high-affinity uptake system. The protein is similar to ChvE in *Agrobacterium tumefaciens* which is necessary for *vir* gene induction when responding to sugars.

1.3.3.2 *Azospirillum* and plant growth promotion

It has been experimentally concluded that *Azospirillum* enhances plant growth. But unlike *Rhizobium* the exact mechanism responsible is still under debate. This is in spite of the fact that intensive research activities are diverted in this direction. Several mechanisms have been proposed to elucidate the plant growth promotion activity of this PGPR including

- Associative nitrogen fixation
- Production of phytohormones (Costacurta and Vanderleyden, 1995)
- Deamination of the ethylene precursor 1-aminocyclopropane 1-carboxylate (Blaha et al., 2006; Holguin and Glick, 2003)
- NO induced lateral root formation (Creus et al., 2005)

It is also observed that inoculated plants absorb minerals and water better than uninoculated control plants. Several metabolic processes are supposed to be affected by the bacteria including cell membrane activity and cell cycle progression. One of the hypotheses called additive hypothesis states that multiple mechanisms rather than a single one is responsible for the increase in plant growth. These multiple mechanisms are suggested to operate simultaneously or in succession. Despite the suggestion of these mechanisms, no definite agreement has been reached upon so far. However, the most

prominent changes after inoculation could be observed in changes of root morphology directly stimulating plant growth. We will therefore discuss these mechanisms with reference to effects of each on root architecture.

Nitrogen fixation

The availability of fixed Nitrogen (N) is often referred to as a major factor hampering crop productivity. Biological nitrogen fixation whereby atmospheric nitrogen is converted into ammonia by symbiotic, associative and free-living bacteria gains a lot of importance in this perspective.

Azospirillum, through the action of the nitrogenase complex, is able to convert atmospheric nitrogen into ammonium under micro aerobic conditions at low nitrogen levels. The assimilation of fixed Nitrogen is carried out through the glutamine synthetase (GS)/glutamate synthase (GltS) pathway (Westby et al., 1987). GS is inactive in N excess and vice versa. The characterization of an N-regulated ammonium transporter (amtB) (Van Dommelen et al., 1998) and studies with the respective mutant clearly indicated the presence of an ammonium uptake system (s) in *A.brasilense.*

Owing to the fact that micro aerobic N-limiting conditions are required for N fixation by *Azospirillum*, all ammonium, glutamine, nitrate and nitrite repress N_2 fixation in *A. brasilense* (Gallori and Bazzicalupo, 1985). Similarly, viewing the sensitivity of the nitrogenase enzyme complex to oxygen, biological nitrogen fixation is tightly controlled both at transcriptional as well as posttranslational level.

Azospirillum in a vegetative form (instead of cyst form) show faster metabolism which directly effects its N_2-fixation capability (Pereg Gerk et al., 2000). The same is apparent from investigations with mutants of the *A. brasilense* strains Sp7 and Sp245. These mutants are defective in flocculation, differentiation into cyst-like forms and colonizing roots. However, they had a higher nitrogenase expression as compared to the wild type strains when associated with wheat.

Using the acetylene reduction assay with excised roots, high rates of nitrogen fixation by *Azospirillum* in association with maize were observed (Von Bulow

and Dobereiner, 1975). The credibility of the findings was questioned as the assay used was reported to overestimate the actual amount of Nitrogen fixed. However, with more credible techniques like ^{15}N dilution and N-balance estimations endorsed in some cases the *Azospirillum* mediated biological nitrogen fixation in plants. Additionally, the characterization of nitrogen fixation genes further supports the existence of the fixation process in *Azospirillum*.

Auxins

The bacteria produce several phytohormones in culture and in association with plants. The three main types of plant growth promoting substances detected in the supernatant of *Azospirillum* cultures are auxins, cytokinins and gibberellins (Bottini et al., 1989; Tien et al., 1979). The auxins indole-3-acetic acid (IAA) quantitatively seems to be the most important of the three phytohormones. Bacterial phytohormone production is assumed to induce changes in root morphology after *Azospirillum* inoculation. The work with *Azospirillum* mutants altered in indole-3-acetic acid production endorses this hypothesis.

As far as biosynthesis of auxin in bacteria is concerned, Tryptophan (Trp) is generally considered as a precursor of IAA, although evidence for tryptophan independent pathways also exist (Prinsen et al., 1993). Three main pathways are documented for the conversion of tryptophan into IAA: the indole-3-pyruvic acid (IPyA) pathway, the indole-3-acetamide (IAM) pathway and the tryptamine (TAM) pathway (Costacurta and Vanderleyden, 1995).

A gene encoding IPyA decarboxylase (ipdC) is shown to be a key enzyme for IAA biosynthesis in this bacterium (Costacurta et al., 1994) as an *A. brasilense* ipdC knock-out mutant synthesized less than 10% of the level of wild-type IAA production. The gene is responsible for the mediation of the conversion of IPyA into indole-3-acetaldehyde (IAAld). This is the only gene isolated and sequenced so far, which is involved in IAA production in *A. brasilense* The involvement of the same gene product is also documented in biosynthesis of the auxin phenyl acetic acid (PAA) having antimicrobial activity (Somers et al., 2005).

The plant growth promoting effects of *Azospirillum* are mainly attributed to the production of phytohormones. When filter-sterilized culture supernatants of *A.brasilense* were added to hydroponically grown rice roots, increased root elongation, root surface area, root dry matter and development of lateral roots and root hairs, compared with untreated roots were observed. However, higher concentrations of the supernatant resulted in inhibition of root elongation, lateral root development, and even caused nodule-like tumors on the roots (El-Khawas and Adachi, 1999). Comparable results were obtained with the application of supernatant of *A. brasilense* Cd to soybean plants significantly inducing root number and length (Molla et al., 2001). Another study involving mutants with low phytohormones capability and high nitrogenase activity further substantiates auxin as a key agent in the plant growth promotion phenomenon. The said mutants failed to enhance root growth over uninoculated partner. Besides, a mutant with increased phytohormones production markedly affected root morphology (Kundu et al., 1997).

The effect of *Azospirillum* inoculation in terms of growth promotion or inhibition is greatly dependent on the inoculums concentration. It is interesting to note that *Azospirillum* mimics the typical growth response induced by auxins, which are inhibitory to plant growth at high concentrations and stimulatory at lower levels. Additionally, the morphological changes induced in the roots independently by auxin and the said bacteria share many similarities. (Dobbelaere et al., 1999).For instance when the same authors, in a plate-assay protocol inoculated seedlings with the wild-type *A. brasilense* strains, Sp245 and Sp7, and with *ipdC* mutants, they noted that with the increase in concentration of the wild type strains, a strong decrease in root length but an increase in root-hair density was observed. Similarly inoculation with the ipdC mutants only inhibited the root length when used in very high concentration (up to 10^9cfu/ml).

Ethylene

Ethylene is well known for its role in breaking the dormancy of seeds during germination. At the same time higher levels of ethylene have been observed of having an inhibitory effect on root elongation. A strategy therefore, resulting in

lowering its levels is of great importance in crop plants. PGPR, including *Azospirillum*, resort to an enzymatic system capable of degrading the precursor of ethylene synthesis namely 1-aminocyclopropane-1-carboxylic acid (ACC). The degrading bacterial enzyme is accordingly called ACC deaminase.

In case of *Azospirillum* previous studies indicated a lack of deaminase activity (Holguin and Glick, 2003). But later on, when a wide range of strains from different species were tested, the presence of not only the corresponding gene (in most strains studied) but also the deaminase activity for few strains of *A.lipoferum* was established (Blaha et al., 2006). These deaminase+ *Azospirillum* strains are thus able to attenuate the ethylene levels and impart growth promotion on the host plant.

Gibberellins

Gibberellins are plant hormones implicated in growth regulation and different developmental processes. Studies on the effect of *Azospirillum spp* inoculation on pearl millet for the first time attributed growth promotion effects partially to gibberellin-like substances detected in supernatants from *A. brasilense* cultures (Tien et al., 1979). Experimental *in vitro* data further elucidated the growth promotion in plants induced by *Azospirillum* inoculation. The main gibberellin identified from *Azospirillum* culture is gibberellin A3. This type is suggested to be involved in promoting maize growth (Lucangeli and Bottini, 1997).

Cytokinins and Abscisic acid

Very little data exist on the production of cytokinins by soil bacteria. Some *Azospirillum* strains are capable of producing compounds with cytokinin-like activity (Tien et al., 1979), but quite limited as compared to IAA. Similarly scant data relating to abscisic acid (ABA) production by *Azospirillum* is available. One study indicated not only the production of this hormone by the Sp245 strain, but also significantly elevated levels of ABA in plants inoculated with the same *Azospirillum* strain (Cohen et al., 2008). The authors thus suggested the involvement of ABA in growth promotion effects of *Azospirillum*. Similarly, ABA

production is also reported for two other *A.brasilense* strains Az39 & Cd in a chemically defined media (Perrig et al., 2007).

1.3.3.3 *Azospirillum* and plant root morphology

The most profound effects on the plant after *Azospirillum* inoculation are connected to changes in the root morphology. For example, *A.brasilense* is capable of altering plant root architecture as its inoculation promotes lateral and adventitious roots development (Fallik et al., 1994). Plant hormones are suggested as key factors in growth promotion induced by the bacterium (Steenhoudt and Vanderleyden, 2000) whereby they stimulate the density and length of root hairs and root surface area, improving the utilization of water and mineral nutrients (Rodriguez-Navarro et al., 2007) However parallel investigations at the same time provide evidence for an NO-dependent root branching promotion activity on tomato upon *Azospirillum* inoculation both in wild type and mutants with attenuated auxin synthesis capability (Molina-Favero et al., 2008).

The proliferation of root hairs is one of the most prominent effects observed upon inoculation with *Azospirillum*. The phenomenon is not confined to any specific class of plants; rather it covers a variety of plant species like grasses, maize, tomato and even legumes. In case of legumes, for instance, *A. brasilense* exposure to common bean (*Phaseolus vulgaris* L.) enhanced the total root length, projected root area/root dry weight and root length/root dry weight resulting in root systems with longer and thinner roots (German et al., 2000).

The bacteria, besides increasing the number and density of root hairs, also reduce the time for appearance of root hairs, increase the mature root hairs length along with and shortening the distance between the root apex and the region where root hairs start to elongate (Dobbelaere et al., 1999; Harari et al., 1988). The root growth promotion is thought to be related to production of phytohormones by the bacterium as similar changes in the root morphology

have been noticed upon application of combination of plant growth substances (Jain and Patriquin, 1985; Tien et al., 1979).

Higher concentrated inoculums of *Azospirillum* (10^9–10^{10}cfu ml^{-1}) are reported to cause root hair branching. In wheat, for example, these deformations were of two different types, one of equal (the tuning fork-like deformation) and the other of unequal lengths (Patriquin et al., 1983). The role of the phenomenon is currently unknown.

Another root altered morphology observed was elongation of primary roots. Several plant species like wheat (Kapulnik et al., 1985b), tomato (Hadas and Okon, 1987) etc. manifested longer roots after being challenged with *A. brasilense*. The effect on both root hair and root length elongation is dependent on the bacterial concentration applied. Concentrations in the range of (10^3–10^6cfu ml^{-1}) stimulated root elongation, whereas inoculums above 10^7cfu l^{-1}) resulted in retarded roots (Harari et al., 1988; Kapulnik et al., 1985b). Interestingly, the changes in the root seem to rely upon production of IAA by the bacterium as witnessed in the experiments using a mutant strain of *A. brasilense* impaired in IAA production (Dobbelaere et al., 1999). Furthermore exogenous IAA application above a certain threshold induced a response mimicking the higher *Azospirillum* inoculums treatment response (Pilet and Saugy, 1985).

Few *Azospirillum* strains yet adopt another mechanism to bring about root elongation and better nutrients availability thereof by diminution of the ethylene concentration via microbial deamination of the plant ethylene precursor 1-aminocyclopropane (ACC) (Holguin and Glick, 2003). The process is mediated by the bacterial corresponding gene acdS. In this manner conversion of ethylene from ACC will be reduced which in turn will minimize the inhibitory effects of the hormone on plant root elongation.

In addition to the colonization effects on root/root hair length, the significant increase in number and length of the lateral roots have also been observed in a number of plant species. These findings were obtained both in laboratory and field conditions. An example in this regard is the increase seen in total number

of roots per plant as well as the number of roots per tiller in a field experiment with wheat, inoculated with *Azospirillum* (Kapulnik et al., 1987). In a field study involving maize inoculated with *A.brasilense* cd,a significant increase in the number of adventitious roots and the total adventitious-root length was noted (Dobbelaere et al., 2001)

1.3.3.4 *Azospirillum* and biocontrol

Scant studies are available on the disease resistance inducing effects of *Azospirillum* species. However, recently *Azospirillum* sp B510, isolated from rice was shown to induce resistance in host rice plant against rice blast disease caused by *Magnaporthe oryzae* and bacterial blight caused by *Xanthomonas oryzae pv. oryzae* (Yasuda et al., 2009).

The decline of take-all disease (Gaeumannomyces graminis var. tritici) in wheat monoculture is shown to be correlated with the prevalence of root colonizing micro organisms particularly *Azospirillum* (Sanguin et al., 2009), suggesting a role for *Azospirillum* in the process.

P. syringae pv. tomato is the causal agent of bacterial speck disease in tomato plants. *A. brasilense* inoculation along with *P. syringae pv. tomato* significantly reduced the disease symptoms and bacterial population on tomato (Bashan and De-Bashan, 2002).

A. brasilense inhibitory effect of on crown gall formation in dicotyledonous plants caused by *A. tumefaciens* had earlier been reported but the mode of action was unknown. Later on in another study such antimicrobial features, at least in vitro, were attributed to phenylacetic acid (PAA), an auxin like substance produced by *Azospirillum* (Somers et al., 2005). Additionally, the substance is suggested to increase the rhizo competence of the bacterium.

1.3.3.5 *Azospirillum* and plant tolerance to a biotic stress

The role of PGPR inoculation is not confined to enhance the growth of the plants only. Rather the upcoming data strongly support its contribution in making the host plant more tolerant to various abiotic stress conditions. In a study where wheat seedlings were inoculated with *Azospirillum*, it was noticed

that the plant was protected from water stress. The action was suggested to be mediated through changes in the fatty acid and distribution profiles of the major root phospholipids phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (Pereyra et al., 2006). Many more studies reported the effects of *Azospirillum* inoculation on drought resistance of the host plants. For instance, under water deficient conditions, inoculated maize seedlings displayed improved relative and absolute water contents, in relation to non inoculated plants (Casanovas et al., 2002).

Azospirillum inoculation has also proved to be effective in mitigating the effects of salt stress in different plants. In one case, for example, *Azospirillum*-inoculated seeds of lettuce (*Lactuca sativa L., cv.Mantecosa*) exhibited superior germination rates and vegetative growth compared to non-inoculated plants under NaCl stress (Barassi et al., 2006). Such beneficial effects of the inoculation have also been documented for other plants like chickpea, faba beans, maize etc.

Salt stress is shown to affect the nodulation process, as reported for the interaction of legume Phaseolus with *Rhizobium*. When these salt stress plants were exposed to a secondary infection by *Azospirillum*, a prolonged exudation of plant flavonoids compared to *Rhizobium* infection alone was observed, implying an induction of flavonoid genes in the presence of *Azospirillum* (Dardanelli et al., 2008). In yet another study, inoculation with *A. brasilense* was shown to significantly mitigate negative effects on plant growth brought about by irrigation with saline water (Hamaoui et al., 2001).

1.3.3.6 *Azospirillum* and plant improved mineral and water uptake

Inoculation with *Azospirillum* is repeatedly attributed with increased mineral uptake by the plant. In addition to N availability both in the form of nitrate and ammonium, the colonization is demonstrated to improve the uptake of other mineral nutrients such as Rb^+ ,$Fe^{2+/3+,}$ K^+, H_2PO_4 etc. (Lin et al., 1983) . Controversy, however, still exists whether this improved nutrient uptake results from a specific enhancement of the normal ion-uptake mechanism or an outcome of the changes in root morphology of the plant. As far as the later view

point is concerned, higher K^+ and Fe^{2+} uptake and higher H_2PO_4 uptake have respectively been attributed to thicker roots and presence of root hairs (Gahoonia and Nielsen, 1998; Gahoonia et al., 2001).

Mobilization of minerals by plants is also suggested to take place by proton extrusion through the membranes of root cells of the treated plants, which results in acidification of the rhizosphere. The rhizosphere acidification has been shown to increase phosphorous and other macro elements and micro elements uptake, especially in arid lands with high calcium contents. Moreover, the process is also observed in cardon cactus plants (Carrillo et al., 2002). However, for induction of this effect , compatible strains of the bacterium seem to be a prerequisite (Amooaghaie et al., 2002).

On the other hand changes in root architecture after *Azospirillum* colonization lead to improved water uptake, making it thus possible to grow plants in water deficient soils. The field trials with dry land Sorghum indicated better water regime and an increase in the yield , which is suggested to be primarily through improved utilization of soil moisture (Fallik et al., 1994). Investigations on two wheat cultivars under osmotic stress also revealed higher turgor pressure at low water potential. The effect is said to be a reflection of the better water uptake after inoculation that, in turn, is manifested in the form of faster shoot growth in inoculated seedlings (Creus et al., 1998).

In light of the facts mentioned above, it can be concluded that *Azospirillum* enhances plant growth, but unlike Mycorrhizal and *Rhizobium*-legume interaction the exact mechanism responsible for this is still debated. This is in spite of the fact that intensive research activities are diverted in this direction. Still we are largely unaware of the early signaling events involved in the PGPR-plant association and the plant genes underlying these processes. Several facts are however beyond doubt: these bacteria fix nitrogen and produce several phytohormones in culture and in association with plants but the transfer of these products is probably limited and not always detected. Still the growth response is evident. It is also observed that inoculated plants absorb minerals and water better than uninoculated control plants. Several metabolic processes are

supposed to be affected by the bacteria including cell membrane activity and cell cycle progression. The most prominent changes after inoculation could be observed in changes of root morphology.

Recent incoming data suggest that plant pathogens and rhizobia are perceived initially as intruders by the host plant triggering similar responses. The success of the infection, however, depends on the ability of the invader to either block or overcome the defenses built up by the host plant (Soto et al., 2009). Probe into the common signaling events observed in the plants in response to both the beneficial microbes particularly *rhizobia* and the pathogenic microbes is thought to help in revealing the occurrence or non occurrence of such events and their genetic basis in *Azospirillum* plant relationship. With this goal in mind, it looks pertinent to go through the interaction between plant and its pathogenic partner and compare the outcome with the already mentioned plant association events with beneficial micro organisms.

1.4 Plant pathogenesis and mutualism

The lack of circulating cells and sessile nature compelled the plant to rely on local and systemic immune responses (Jones and Dangl, 2006). One branch of the plant immune system makes use of the trans membrane pattern recognition receptors (PRRs) involved in interaction with pathogen associated molecular patterns (PAMPs/ MAMPs.

In order to colonize a particular host, bacteria have to develop the ability to circumvent defensive barriers elaborated by the plant to prevent infection. Once these barriers are breached, the newly susceptible host faces selection pressure to develop countermeasures that block invasion by the pathogen. The plant thus evolves a *de novo* strategy by exhibiting novel resistance responses and compels the pathogen at the same time to respond with an alternative mechanism that could restore its virulence. These dynamic and ongoing co evolutionary battles have resulted in the utilization of highly specific and extremely sophisticated attack strategies by the pathogen and equally elaborate defense responses by the host.

The above illustration describing the dynamics of plant microbe interaction was proposed by Jones and Dangl as a four-phased ‘zigzag’ model (Jones and Dangl, 2006). The first step which is marked by the recognition of PAMPs by PRRs is named PAMP-triggered immunity (PTI). This may halt or limit further colonization. Successful pathogens, on the other hand, deliver effectors that interfere with the initial plant response thus facilitating pathogen nutrition and dispersal. The phase was termed effector-triggered susceptibility (ETS). In the next stage a more robust response from the host is elicited when the NB-LRR proteins recognize certain effectors resulting in effector-triggered immunity (ETI).This particular defense version is usually accompanied by the hypersensitive response resulting in cell death and subsequent resistance to the disease. In the last phase as a result of evolutionary pressure, pathogens manage to escape ETI either by modifying or acquiring new effectors types.
In order to perceive a variety of MAMPs, plants possess diverse PRRs. Although a list of bacterial elicitiors and the corresponding plant receptors are documented, the bacterial flg22-FLS2 (Gomez-Gomez and Boller, 2000) and elongation factor EF-Tu -EFR (Zipfel et al., 2006) elicitor receptor systems are perhaps the best investigated ones. When plants were challenged with purified MAMPs, the early responses recorded were a change in cytoplasmic Ca^{2+} levels, ion-flux across the plasma membrane, apoplastic alkalinization, production of reactive oxygen species (ROS) and nitric oxide (NO), activation of MAPK cascades and the induction of defense genes in many plant species (Nurnberger et al., 2004). Strikingly quite few of these responses are also shared by epidermal cells of legume roots soon after application of Nod factors (Felle et al., 2000; Ramu et al., 2002). However, in case of mutualistic interaction robust defense responses are not observed. It is of interest to note that bacterial flagellins which are perceived by an array of plant species, are inactive as MAMPs in *Arabidopsis* when derived from rhizobia (Gomez-Gomez et al., 1999), however EF-Tu, despite its perception, is unable to elicit a defence response in legumes (Kunze et al., 2004).
In a plant pathogen interaction, in addition to local defence responses such as the HR, plants can also develop systemic defences which are spread

throughout the plant organs. This type of reaction, which in the plant pathogen perspective is termed systemic acquired resistance (SAR), is triggered after a first infection by an incompatible necrotizing pathogen that subsequently results in a more resistant host plant to further attacks by virulent pathogens (Durrant and Dong, 2004; Sticher et al., 1997). A large number of reports indicate the occurence of such a systemic immunization against a wide range of plant diseases even when plants are exposed to PGPR and is called induced systemic resistance (ISR) (Bakker et al., 2007; Kloepper et al., 2004; van Loon et al., 1998). Although ISR related signal transduction is well studied, molecular mechanisms underlying the early interaction events including perception of ISR-inducing bacterial elicitors by the plant cell are relatively less investigated. In one of such studies the lipopeptides (LP) elicitor of *Bacillus subtilis* induced defense-related early events in Tobacco cells such as extracellular medium alkalinization coupled with ion fluxes, reactive oxygen species production and stimulation of a phenylalanine ammonia lyase and a lipoxygenase (Jourdan et al., 2009).

Similar findings are shown for few other PGPRs like *P. fluorescens* (van Loon et al., 2008). In the later case, increases in the cytosolic calcium levels were also observed. The findings provide not only an insight into the way elicitors from beneficial bacteria can be perceived by the host plants but also reveal the commonalities in the early events after recognition of the elicitor by host plant cells between the pathogenic and beneficial bacteria.

1.5 Aims of the project

The current project aims at recognizing the underlying molecular processes involved in the interaction of the diazotrophic soil bacterium *Azospirillum brasilense* with its host plant *Arabidopsis thaliana*. Here we will focus on (i) a comprehensive overview of *Arabidopsis* genes that underlie regulation upon association with *A. brasilense*, (ii) to identify individual genes as essential elements for the plant growth promoting effect and (iii) to characterize the bacterial signals that regulate plant gene expression. An attempt was, therefore, made to characterize the early responses of plants upon association with *A. brasilense* by:

- setting up a pH-monitoring system that allows us to follow the amplitude and kinetics of elicitor induced pH changes in the apoplast of plant cells.
- A luminol based ROS production assay
- Universal transcriptome profiling of *Arabidopsis thaliana* seedlings upon inoculation with *Azospirillum* brasilense strain Sp7.

2 Material and methods

If not mentioned otherwise, all chemicals are from Sigma (Sigma-Aldrich, Steinheim, Germany) or AppliChem (AppliChem, Darmstadt, Germany).

2.1 Plant material and growth conditions

2.1.1 For microarray

Arabidopsis thaliana accession Columbia-0 (Col-0) seeds were initially surface sterilized by immersing them into 100% ethanol and leaving on rotary shaker (A.Hartstein) for 20 minutes. After removing the ethanol, 1 ml of 6% Sodium hypo chloride containing 0.2 % triton was added to the same 1.5ml tube and left on rotary shaker for not more than five minutes. Seeds were then washed five times with sterilized water under the clean bench. The dried seeds were then seeded on petri dishes containing Sussman medium (table2) with 1.5 % agar Kobe 1 supplemented with 1% sucrose. Prior to platting media in petri dishes pH was adjusted to 5.7 with KOH and then autoclaved. The plates were sealed with Dura seal/ micropore tape and placed at 4°C for 72 hours to allow them to vernalize and imbibe. After which they were placed in the growth chamber having long day conditions of 16 hours light(22°C) 8 hours dark(16°C).The sterilized conditions were maintained in order to ensure the effects only from the experimental organism. The plates in the chamber were vertically oriented so that roots could grow on the surface of the media.

2.1.2 For Reactive species (ROS) measurements

Arabidopsis thaliana accession Columbia-0 (Col-0), its T-DNA insertion line elongation factor receptor (*efr*) mutant and *Arabidopsis* ecotype Wassilewskija (*Ws*) were grown in pots for six weeks. Round leaf discs of the size of the well of 96 well plate were cut and incubated over night in sterilized water. Next day they were incubated in 50µl of reaction mix (2oµl/ml each of luminal 20mM and

Horse reddish peroxidase 1mg/ml) in 96 well plate for 30 minutes and then assayed for different treatments.

Item	Amount
Potassium nitrate (KNO_3)	5mM
Calcium Nitrate $Ca(NO_3)_2$	2.5m
Sodium dihydrogenphosphate dehydrate NaH_2PO_4 $2H_2O$	2mM
Magnesium sulphate hepta hydrate $MgSO_4$ $7H_2O$	2mM
FeNa EDTA	0.1mM
Calcium chloride ($CaCl_2$)	25μM
Boric acid (H_3BO_3)	25μM
Zinc sulphate hepta hydrate ($ZnSO_4$) $7H_2O$	2μM
Mangenese Sulphate hepta hydrate ($MnSO_4$) $7H_2O$	2μM
Copper sulphate penta hydrate ($CuSO_4$) $5H_2O$	0.5μM
Sodium molybdate dihydrate (Na_2MoO_4 $2H_2O$)	0.2μM
Cobaltous chloride ($CoCl_2$)	0.01μl
Sucrose	1%

Table 2 Constituents of sussman media used for growing *Arabidopsis* seedlings on agar plates.

2.1.3 For *Arabidopsis* and tomato cell cultures

Arabidopsis and tomato cell cultures were maintained in their respective growth media (pH5.7) (table3) on the shaker (Infors AG, CH 4103 Bottmingen, Switzerland) at 24°C and 120 rotations per minute (rpm). *Arabidopsis* and tomato cells were used in experiments after four and five days of sub cultures respectively.

Item	Tomato	*Arabidopsis*
Murashige & Skoog (1X)	4.9g/lit.	4.9g/lit.
Sucrose	30 g/lit.	20g/lit.
Kinetin	50ml (0.4g/l stock)	
2,4 D	10μl/lit. (0.2g/l stock)	10μl/lit. (0.2g/l stock)
KH_2PO_4	0.17g/lit.	
Ferrus solution (0.7g $FeSO_4.7H_2O$+0,95g EDTA)		20ml/lit

Table 3 Constituents of tomato and *Arabidopsis* cell culture media.

2.2 Bacterial cultures and elicitor preparations

2.2.1 Bacterial cultures

Azospirillum brasilense strain sp7 and *Agrobacterium tumefaiciens* strain C-58 cultures destined for elicitor preparations were grown overnight in 250ml erlenmeyer flask containing Kings broth medium (table 4) in an incubator shaker (Innova 4230, New Brunswick Scientific Edison,NJ,U.S.A.) at 28°C rpm140.

Item	Amount
Peptone	20g/lit.
Magnesium Sulphate hepta hydrate $MgSO_4\ 7H_2O$	1.5g/lit.
diPotassium hydrogen phosphate K_2HPO_4	1.5g/lit.
Glycerol 87%	10ml

Table 4 Kings broth medium

Similarly *Azospirillum brasilense* strain sp7 when used for inoculating the plants for processing in the microarray experiments, they were grown in MMAB media (table 5). Sp7was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ).

Item	Amount (ml)
10% diPotassium hydrogen phosphate K_2HPO_4 (Water free)	30.0
10% Sodium dihydrogen phosphate NaH_2PO_4	10.0
10% Ammonium chloride NH_4Cl	10.0
10% Magnesium Sulphate hepta hydrate $MgSO_4\ 7H_2O$	3.0
10% Potassium chloride (KCl)	1.5
1% Calcium Chloride ($CaCl_2$) x 2 H_2O	1.0
o.1% Ferrus sulphate hepta hydrate $FeSO_4\ 7H_2O$	2.5
10% Na-Malat (7.5 g DL-Malat + 4.48 g NaOH)	50.0
0.1% Biotin dissolved with KOH	5.0
10% Trace element solution SL-6 from DSMZ	0.2

Table 5 MMAB medium

Item	grams/liter
Zinc Sulphate hepta hydrate ($ZnSO_4$) . $7H_2O$ $ZnSO_4$ 7 H_2O	0.10
Manganese Chloride Tetrahydrate. $MnCl_2$ x 4 H_2O	0.03
Boric acid (H_3BO_3)	0.30
cobalt dichloride hexahydrate $CoCl_2$ 6 H_2O	0.20
Copper chloride dehydrate $CuCl_2$ 2 H_2O	0.01
Nickle chloride hexahydrate $NiCl_2$ 6 H_2O	0.02
sodium molybdate hydrate Na_2MoO_4 H_2O	0.03

Table 6 Trace elements solution used in sussman medium

2.2.2 Elicitor preparations

2.2.2.1 Flagella isolation

Azospirillum brasilense strain sp7 and *Agrobacterium* C-58 were grown in Kings Broth medium overnight under the same conditions mentioned above and spread on KB plates containing 1.5 % agar Kobe1 after concentrating them by centrifugation. The following steps were then carried out:

- Bacteria were grown there for 1-3 days; cells were scraped off from plates and resuspended carefully in 15ml (total) Tris-Cl buffer (20mM, pH 8).
- Flagella were sheared off on ice with Kinematica Polytron Blender (level 5, 5 min).
- Bacteria were centrifuged at 10.000xg, 4°C, for 30vmin
- Pellet was stored at -20°C for further processing later on.
- While stirring on ice added solid $(NH_4)_2SO_4$ slowly to a final concentration of 20% (w/v) to the supernatant.
- At 4°C mixed over night on a rotary shaker (overhead).
- Performed centrifugation (20.000xg, 4°C, 30 min.) and resuspended pellet in 300µl HEPES-BTP buffer (0.5mM, pH 7).
- Dialyzed (10000MW) against HEPES-BTP buffer
- Aliquot dialized proteins (~50µl) and stored at -80°C.
- Checked quality and confirmation of protein of interest in 12% SDS PAGE.

2.2.2.2 Bacterial lysates

The pellet obtained after the 10.000xg step during the procedure adapted for isolation of flagella was further processed for getting bacterial lysates. Following steps enabled us to get lysed bacteria:

- Resuspending the pellet completely in HEPES-BTP buffer.
- Sonification for 30 sec (4 xs) with the interval of 30 sec on ice (Bandelin Sonoplus, Berlin, Germany).
- Centrifugation at 14000xg for 20 minutes at 4°C.
- Taking the supernatant, making aliquotes and storing at -20°C.

2.2.2.3 Live bacteria

Bacteria were grown up to 1.2-1.5 OD_{600}, washed and resuspended in the media used for growing cell cultures. The bacteria on both occasions were pelleted at 2250 x g for 15 minutes at room temperature. The volume of media used for re suspension was 200µl.

2.3 Bacterial DNA isolation and PCR conditions

Overnight grown bacterial culture was used to isolate the DNA. !ml of the bacterial culture was centrifuged. The pellet after washing was resuspended in 100µl of water and incubated at 95°C for ten minutes. The released DNA was obtained after pelleting the debris.

The DNA was amplified using the following primers.

EF-Tu. deg-fwd; ATGGCDAARRVHAARTTYGADCG

EF-Tu. *Azospirillum* B510-rev; GTGATCATGTTCTTCACATAGTC

The PCR conditions are summarized below:

Initial denaturation	5’	95°C
Denaturation	30’’	95°C
Primer-annealing	30’’	56°C
Elongation	45’’	72°C
Final elongation	10’	72°C

cDNA (~10ng)	1µl
dNTPs (10mM)	0.4µl
sense Primer (10µM)	1µl
antisense Primer (10µM)	1µl
10x Puffer	2µl
Taq DNA-Polymerase	0.2µl
$MgCl_2$	0.8µl
Rest	add PCR water to make total volume 20µl

PCR product, after verification on gel documentation system was sequenced in an in-house ABI Prism 3100 Avant Genetic Analyser (Applied Biosystems, Karlsruhe, Germany). Sequence comparison was done using NCBI online freely available BLAST/blastx program (http://blast.ncbi.nlm.nih.gov).

2.4 Inoculation of plants

Bacteria, grown overnight in MMAB media, were centrifuged in a 50 ml falcon tube at 2250 g for 15 minutes at room temperature. The brakes and acceleration were adjusted at 5.The pellet was then completely washed and re suspended in the media used for growth of plants. Optical density (OD_{600}) was adjusted to 1. It was then used to inoculate the *Arabidopsis* plants @200-300µl/plate. These seedlings were later used for isolation of RNA for use in microarray experiments.

2.5 Bioassays

2.5.1 Medium alkalinization responses

The extracellular alkalinization of the growth medium (the alkalinization response), upon elicitation was continuously measured using a small combined glass electrode (Knick, Berlin, Germany). About 4-5ml aliquots of the cell

suspensions were placed in open 20ml vials on a rotary shaker at room temperature and with 100 cycles per min. Using Profilab 4.0 pH recorder software, changes were recorded every second.

2.5.2 Reactive Oxygen species

Production of hydrogen per oxide (H_2O_2) after treatment of plant material with different elicitors was measured on a 1450 liquid scintillation counter and luminescence counter (Perkin Elmer precisely). As described earlier the leaf discs incubated in a 50µl eaction solution containing 20µl/ml each of Horse reddish peroxidases 1mg/ml) and 20mM luminal were challenged with dispensing volume of 50µl of the same reaction solution also containing the elicitor this time. Chemiluminescent signals were recorded in the form of counts per second (CPS1) for 45 cycles.

2.6 RNA isolation

RNA was isolated using E.Z.N.A, plant RNA mini kit (Omega bio-tech, GA, USA). First of all 75-100mg seedling samples were collected in a 1.5ml micro centrifuge tube. Two clean iron small balls were put into each tube and the tubes were put into liquid nitrogen. The frozen samples were ground/pulverized in a ball mill at 30 rpm for 2 minutes. 500µl of Buffer RB containing β-mercaptoethanol was then added to the sample followed by vigorous vortexing. The supernatant was transferred to an Omega homogenizer column and centrifuged 14,000 x g for 5 minutes. The supernatant of the flow through was transferred to 1.5 tubes and centrifuged again for 30 minutes at 4ºC. The supernatant and an equal amount of 70% ethanol were vortexed mixed in a new tube for 20 seconds. This mixture was transferred to HIBind RNA mini column and centrifugation was done at 12,000 x g for 1 minute. Discarded the flow through and added 500µl of RNA wash buffer1 to the same column and centrifuged at 10,000 x g for 30 seconds. Again discarded the flow through and added 700µl of RNA wash bufferII (buffer was diluted with absolute ethanol before use) to the HIBind RNA mini column and centrifugation step performed

at 10,000 x g for 30 seconds. A second washing with 500µl of RNA wash bufferII and centrifugation thereafter carried out at 10,000 x g for 30 seconds. Discarded the flow through and then column was dried by putting it in the empty collection tube and centrifuging it at full speed for 2 minutes. For eluting RNA the column was transferred into a clean 1.5ml tube and 30µl DEPC water was directly applied onto the matrix of the column. Centrifugation carried out at 10,000 x g for 1 minute. This step was repeated in order to recover the maximum RNA from the column. All centrifugations except one were carried out at room temperature.

2.6.1 Total RNA concentration

To check the concentration of the total RNA, from each sample 1µl was added to 69µl of DEPC water. Using 70µl DEPC water as reference, total RNA concentration was measured with the help of spectrophotometer GeneQuant pro (Ge-healthcare).

2.6.2 RNA gel

Before processing the samples for microarray analysis, the quality of each sample was ensured on RNA gel. 1% Pea agarose in 25ml of 1X MEN buffer was heated in the microwave and then left to cool to about 60°C. At this stage 4.5µl of Formaldehyde was added. !-2µl of RNA sample along with 4µl Loading buffer and 2µl of DEPC water was loaded on the gel after incubation at 65°C for 5 minutes and subsequent cooling at ice for 2 minutes. The results were checked under the gel documentation system.

2.7 Microarray Hybridization and stastical analysis

Microarray chips (Agilent Technologies, Palo Alto, CA) were hybridized to RNA, isolated from 2-week-old Col plants, at University of Erlangen Nürnberg. The obtained raw data were analyzed with different R packages from the Bioconductor project (www.bioconductor.org) at Institut für Hygiene und Mikrobiologie, Universität Würzburg, Germany. Differentially expressed genes

were sorted for the earlier time points (6hpi &24hpi), with the adjusted p values cut off levels of 0.05 for6hpi &24hpi and 0.01 for 96hpi. Gene ratios on the log scale are determined with log base 2.

2.8 DNA digestion

For a 20µl reaction 3µg total RNA from each sample was incubated at 37°C for 30 minutes alongwith 0.5µl RNase inhibitor, 1µl DNase1 and 2µl of DNase 1 buffer. The volume was filled upto 20µl with DEPC water. After the incubation another 80µl of DEPC water was added to each reaction to make total volume to 100µl. We then added 70µl of isopropanol RNA grade, 10µl of 5M ammonium acetate dissolved in 100mM EDTA and 1µL glycogen and centrifuged for 25 min at 14000 rpm at 4°C.

After discarding the supernatant, 500µl of 70% RNA grade ethanol was added to each sample and centrifugation under the same above mentioned conditions was carried out but this time for 20 minutes. The pellet thus obtained was placed was placed at 37°C for 1-2 minutes to get rid of the remaining ethanol, if any. In the last the RNA was eluted with 7µl of DEPC water

2.9 Reverse transcription

cDNA was generated by incubating the isolated total RNA in Landgraf thermo cycler alongwith RT primer oligo dt, dntps and RT puffer for 2 minutes at 70°C. The volume of each used with concentration is shown in the following table. After incubation 0.4µl of reverse transcriptase (MMLV Promega) was added to it and was then placed in Landgraf thermocycler for 1 hour at 42°C.

Item	Amount
Total RNA	6.7µl
RT primer oligo dt 100µM	0.4µl
Dntps 10mM	0.5µl
5x RT puffer(Promega)	2.0µl
MMLV RT	0.4µl
Total	**10µl**

Table 7 cDNA synthesis from total RNA; amounts of items used.

2.9.1 Gel electrophoresis

3% Agarose gel was prepared in 1X TBE buffer(250ml) and added to it 4 µl of Ethidium Bromide. 10µl of the samples containing 8µl probe and 2µl 5x DNA loading buffer were loaded on the gel and visualized.

2.10 Real time qRT-PCR

2.10.1 Primer designing

To validate the microarray data on real time quantitative PCR, fifteen candidate genes among the differentially regulated ones for different time points were selected. Primers were designed using Light Cycler Probe Design software, version 1.0 (Roche Applied Science) and alternatively Oligo, Primer analysis software 6.63 (Molecular Biology Insights, Inc. U.S.A). While designing the primers following points were viewed:

- cDNA sequences were used.
- Preferred length of the primer was 17-18bp.
- C terminus sequences selected for designing.
- Tm of the primer was kept at 54-56°C.

Each member of the primer pair was checked for its specificity to the gene in question by Blast at **http://www.arabidopsis.org/Blast/.**The table 8 below gives the primers sequences, their length and the product size.

AGI	Forward primer(FP)	FP (bp)	Reverse primer(RP)	RP (bp)	Product (bp)
At1g08090	TAGTCGCTTGCACGTTAC	18	ACATTGTTGGGTGTGTTC	18	244
At1g26380	TATACCACATGGTTGGAC	18	CTCCTCACCCTTGATAAA	18	299
At2g18690	CTCTCAGTCCTATTTTGCT	19	TCTTCCCATATTGATCAG	18	199
At2g42060	TTACAGCGCTGGTACCTT	18	CTGGATTGGGCTTATTCA	18	326
AT2G3383	CTCCGCAATAAGATCACC	18	CGCTGTATAGCCAATCG	17	244
At3g54040	AATCTGCCAACAAATATG	18	AATGTTAGGGCAATAGT	17	267
At3g57540	GTGACGACTGCTTCGGTG	18	GCCTTCGCTCTCCTATCT	18	206
At3g49700	CTTGTCTTAAAAGCAACG	18	CTATCTGTTGACTCTACG	18	250
At3g02040	GAGGAACTGAGATGTACC	18	GTTGTATCAACTCGGGA A	19	378
AT3G13610	TTAACTACTACCCCATCT	18	AATACTTGACGTAATCAG	18	384
At4g07820	AGACACTGACTGCCTATG	18	CACTAAGAATGACAGAC GG	19	315
AT4G14630	TGTTCGTATAGACTATGC	18	GCTTGTAAATTCCTGACC	18	363
At4g25250	ATGAAGTATGTCAGAGGC	18	GGCTAAAGTATTGCTTGT	18	195
At5g59680	GTACTCCTGGTTACCTTG	18	CAGGGGAACTTAGTGTA T	18	372
AT5G41300	TCACGCCGTATTATTACT	18	CTATTTCCTCTAATGGAG C	19	271
At5g44420	TCATCACCCTTATCTTCG	18	TTGGCTCCTTCAAGGTTA	18	163
AT5G45070	GAGTGCTATCTATGACAG	18	CAATTTTCTCCTATGTAT	18	270

Table 8 Deatis of primers used in qRT-PCR; showing primer sequence, length and the product size for each gene.

2.10.2 Determination of annealing temperatures of primers

The annealing temperature best suited to a particular selected primer was determined either with the normal PCR or with the help of temperature gradient run for remaining primers on Realplex. In both cases cDNA was used as a template.The normal PCR was run according to the following specifications:

Initial denaturation	1’	95°C	
Denaturation	30’’	95°C	
Primer-annealing	30’’	54°C/56°C	
Elongation	45’’	72°C	**40 cycles**
Final elongation	6’	72°C	

20µl reaction for the PCR was formulated as under:

cDNA (~10ng)	0.5µl (1:20 dilution)
dNTPs (10mM)	0.4µl
sense Primer (10µM)	0.5µl
antisense Primer (10µM)	0.5µl
10x/5x Puffer	2µl/10µl
DNA-Polymerase	0.2µl
DMSO	5%
Rest	add PCR water to make total volume 20µl

Temperature gradient function of the real-time PCR was performed on a Master cycler ep realplex (Eppendorf, Hamburg, Germany).

A 20µl reaction in qRT- PCR contained:

Primer mix 6	8µl
SYBR green dye	10µl
cDNA(1:20)	2µl

Primer mix 6 was formulated by mixing 6µl each of 50µM forward and reverse primers with 388µl of HPLC grade water.

2.10.3 Gel documentation

In both normal PCR and Realplex, PCR product was processed by adding to it 4.5µl of 5X DNA loading buffer, running on 3% agarose gel and visualization under the gel documentation system.

2.10.4 PCR Product Purification

The PCR product was purified using QIA Quick PCR Product Purification kit and the protocol described therein. The three replications for each primer were pooled together and added to that 5 volume of Buffer PB and mixed them (e.g. for 50µl PCR product 250µl of buffer).The samples were then applied to the column and centrifuge for 45 seconds at full speed at room temperature. Then 700µl of PE buffer was applied to the column and centrifuged for 45 seconds. After drying the column by centrifugation for 1 minute and placed the column in 1.5ml tube and add to it 30µl of HPLC water and centrifuge for 1 minute. This step is carried out twice.DNA concentration of the samples were measured by spectrophotometer.

2.10.5 Spectrophotometery

Concentration of various samples was determined on a nanodrop spectrophotometer (IMPLEN).

2.10.6 Making of Standards1-10 for qRT-PCR

A known quantity of cDNA was taken to make serial dilutions of it. Reference standard curve was constructed for standard 7-10 on Light cycler as per already described PCR program. The series of dilutions is described below:

Std 1	10ng/µl of cDNA	
Std 3=	100pg/µl	2µl of std 1+198µl of tRNase free water
Std 5 =	1pg/µl	2µl of std 3+198µl of tRNase free water
Std 7=	10fg/µl	3µl of std 5+297µl of tRNase free water
Std 8=	1fg/µl	20µl of std 7+180µl of tRNase free water
Std 9=	0.1fg/µl	20µl of std 8+180µl of tRNase free water
Std 10=	0.01fg/µl	20µl of std 9+180µl of tRNase free water

Standards 7 to 10 were used in the Realplex PCR as reference standard curve to know quantitative information for mRNA targets of unknown concentrations.

2.10.7 Buffers

Item	Amount
Bromphenol blue	10µl
MEN-buffer	160µl
Formaldehyde	260µl
Glycerin (80%)	100µl
Formamide	720µl
dd H2O	250µl
Ethidium bromide	1µl

Table 9 RNA probe buffer

Item	Amount
MOPS	200mM
EDTA	10mM
Sodium acetate	50mM

Table 10 10X MEN buffer

Item	Amount
Bromphenol blue	0.25%
Xylencyanole	0.25%
EDTA	100mM
Glycerol	50%

Table 11 5X DNA probe buffer

Item	Amount
Tris	0.9M
Boric acid	0.9M
EDTA	20mM

Table 12 10X TBE buffer

3 Results

3.1 Early signalling events

3.1.1 Extracellular alkalinization and PAMP perception

The ability of suspension-cultured plant cells to respond to various microbial elicitors has made them a system of choice to study elicitor perception and elicitor responses. Among the earliest responses towards elicitors, extracellular alkalinization, which itself represents an outcome of altered ion fluxes across the plasma membrane, is considered as a credible, swift and conveniently recordable physiological response to microbe associated molecular patterns (MAMPs) in plant cell cultures (Boller et al., 1995; Nurnberger et al., 2004). Using the well characterized flagellin/FLS2-receptor system as a positive control we investigated the susceptibility of *Arabidopsis* suspension-cultured cells towards different fractions of lysed *A. brasilense* and *A. tumefaciens*

3.1.1.1 Validation of the system

Flg22-Δ2 (a peptide shortened by 2 amino acid residues at the C terminus) is known for being an antagonist to flg22 when added to cells of *A. thaliana* in concentration up to 30 µm (Bauer et al., 2001). Taking advantage of characteristic feature of flg22-Δ2, we tested working of our system using it in combination with 4nM flg22 in *Arabidopsis* cell cultures.

Due to limiting amounts of the antagonist we, however, used Flg22-Δ2 in concentrations upto 7.5µM only. The results as depicted in Fig 1 indicated a significant reduction in extracellular alkalinization of the cells in comparison to flg22 when the antagonist flg22-Δ2 was used in high concentrations. At lower concentrations, the response of *Arabidopsis* suspension cell cultures was not different from flg22 alone. The behavior of the cells both towards flg22 and flg22-Δ2 demonstrated the functionality of our pH-recording system.

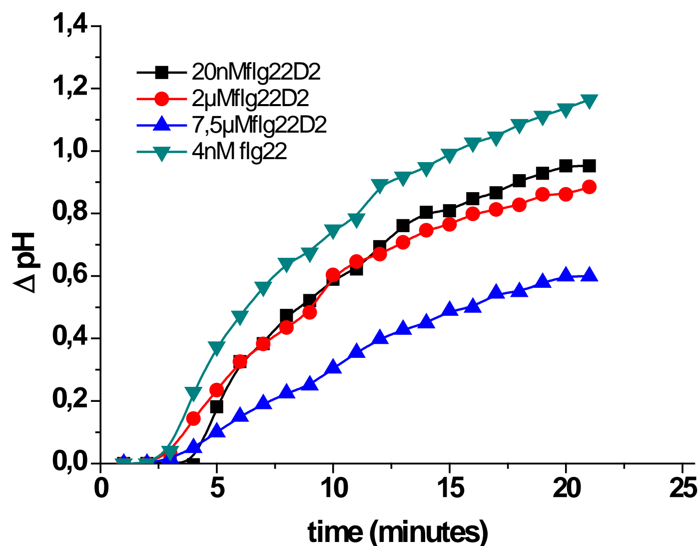


Figure 1 **The antagonistic activity of peptide flg22Δ2 (D2).** Different concentrations of the antagonist were used to observe its effect on induction of medium alkalinization in *A. thaliana* cells as compare to the effects of flg22 alone

3.1.1.2 *Azospirillum* flagella do not induce extracellular alkalinization

In order to test whether flagellar proteins from *Azospirillum* would represent recognizable MAMPs such as flg22 from Pseudomonas we isolated the flagella from *A. brasilense*, and A. *tumefaciens*, and tested their ability to induce extracellular alkalinization response in suspension cultured cells of *Arabidopsis thaliana* and Tomato. These experiments showed that in suspension cultured cells, the isolated flagella from *Azospirillum* and *Agrobacterium* (data not shown) were completely inactive and did not elicit a pH response in terms of extra cellular alkalinization. Such a response for *Agrobacterium* is in line with the already documented results which proved an elicitation activity for the elongation factor EF-Tu but not flagellin proteins (Kunze et al., 2004).

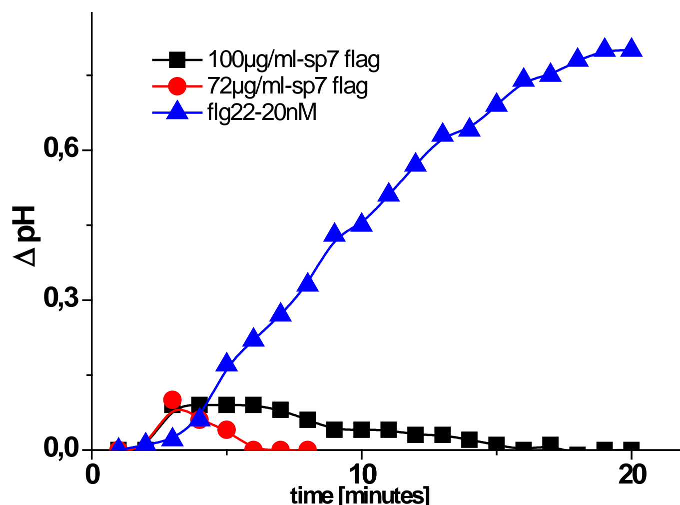
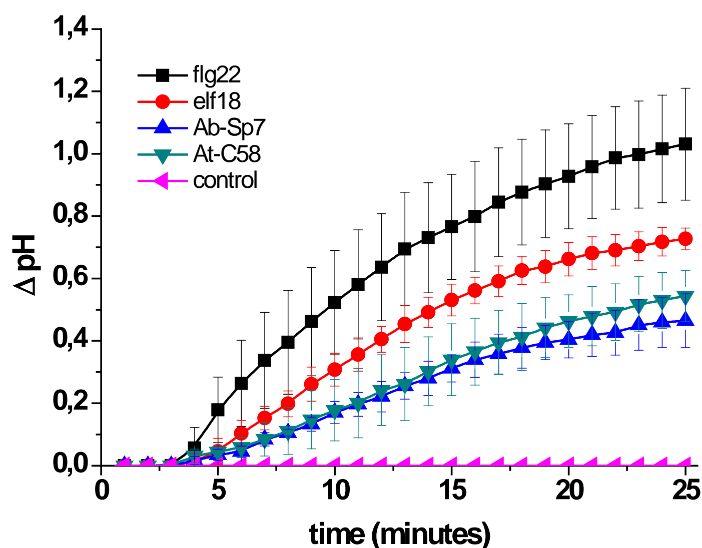


Figure 2 **The inactivity of flagella isolated from *A.brasilense*.** Representative traces of different concentrations of the flagellar protein from *Azospirillum* were used to observe their effect on induction of medium alkalinization in *A. thaliana* cells as compare to the effects of flg22

3.1.1.3 *Azospirillum* lysates and medium alkalinization.

Lysates of *Azospirillum* Sp7 was then tested for apoplastic alkalinization in the *Arabidopsis* cell cultures. Unlike the flagella, the Sp7 preparations generated an alkalinization response which started after a lag phase of 6-7 min. The activity of these lysates was highly reproducible and obtained with any bacterial extracts prepared independently. Comparable results were obtained when *Arabidopsis* cell cultures were challenged with *Agrobacterium* C-58 lysates.

A comparative study of the elicitor activity of these extracts along with flg22 and elf 18 is summarized in the following figures (fig 3). During the course of these experiments it became evident that flg22 had the highest activity in terms of pH change of the extracellular milieu followed by elf18. The two bacterial extracts from *Azospirillum* and *Agrobacterium* exhibited comparable activity in the suspension cell cultures.



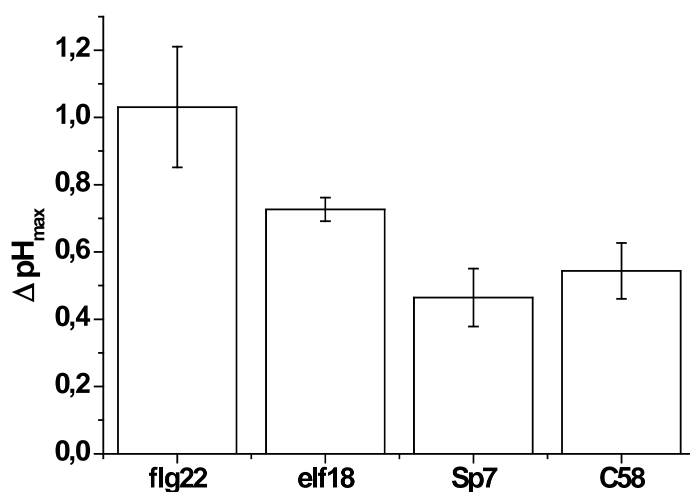


Figure 3 **Comparative elicitation activity.** Data showing comparison of the apoplastic alkalinization potential of two known bacterial elicitors with lysates from Sp7 & C-58. Hepes buffer in which bacterial extracts were washed and resuspended was used as a mock treatment. Both lysates were used at 5µg/ml, while the concentration each of flg22 and elf18 was 20nM. Data represent mean values of n = 6 ± SD

3.1.1.4 Nature of elicitor

Once we came to know that the elicitor active portion of *Azospirillum* resides in the bacterial segments other than flagella, we turned our attention towards exploring the nature of that particular elicitor. Incubation of bacterial extracts from Sp7 with the unspecific protease Pronase drastically reduced the activity in cell cultures. These results suggested that the factor in *Azospirillum* bacteria eliciting alkalinization in *Arabidopsis* cells is proteinaceous in nature (Fig 4).

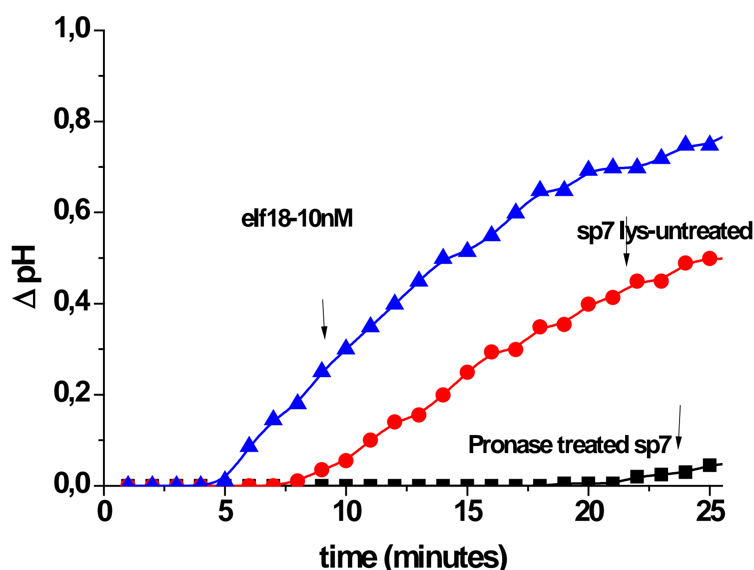


Figure 4 **Protease treatment reduces elicitor activity of *A. brasilense* lysates.** Representative traces of extracellular pH observed in *Arabidopsis* cells when challenged with elicitor elf18 & crude bacterial extracts from *A.brasilense* strain Sp7. The extracts were applied at the rate of 5µg/ml with and without pronase pre incubation (100 mg/ml, 15 min, and 25°C).

3.1.1.5 Activity of *Azospirillum* lysates in tomato cells

Tomato represents a long standing model system to study the interaction of *Azospirillum* and plants. To prove whether the molecular mechanism of *Azospirillum* lysates recognition is conserved between *Arabidopsis* and tomato, we investigated the extracellular alkalinization inducing effect of Sp7 lysates in tomato cell cultures.. The tomato cells by virtue of their sensitive chemo perception system for conserved flg22, demonstrated a prominent response to

flg22 but not to elf18. This already known behavior served as a control in our studies. Interestingly the bacterial preparations from Sp7 could not elicit any medium alkalinization response in tomato cells. Similarly *A. tumefaciens* extracts, which have already been shown being inactive in the tomato cell suspension system(Felix et al., 1999), manifested the same inactivity in our studies with tomato cells. This trend compelled us to duly consider the elongation factor protein as a candidate for elicitor activity in *Azospirillum brasilense* lysates during interaction with *Arabidopsis* (Fig 5).

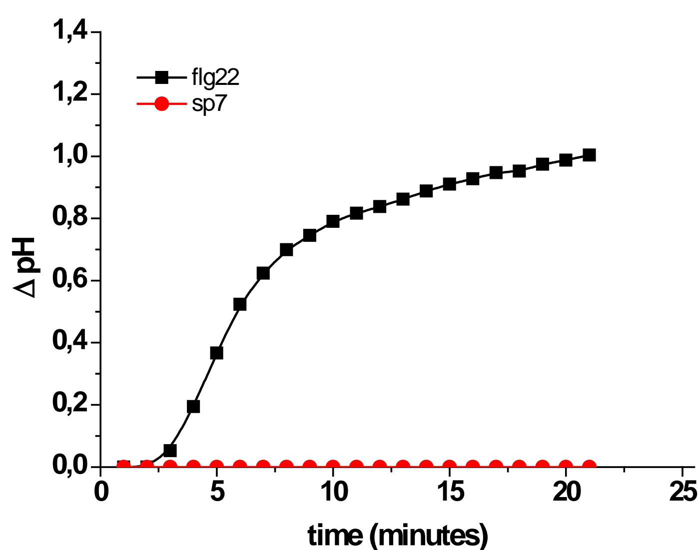


Figure 5 ***Azospirillum* lysates inactivity in tomato cell culture**. Representative traces of tomato suspension cell culture response in terms of pH change to bacterial extracts from *A.brasilense* strain Sp7 (10µg/ml) and 4nm of flg22

3.1.2 Reactive oxygen species (ROS) production

3.1.2.1 Validation of the experimental system

Flg22, a twenty two amino acid long peptide from *Pseudomonas spec*. which corresponds to the most conserved domain of eubacterial flagellin acts as a potent elicitor inducing oxidative burst, among other defense responses, in cells of different plant species (Felix et al., 1999). However, *Arabidopsis* ecotype Ws-0 is insensitive to perceive flg22 due to mutation in the kinase domain of its flagellin receptor FLS2 (Gomez-Gomez et al., 1999). Similarly,

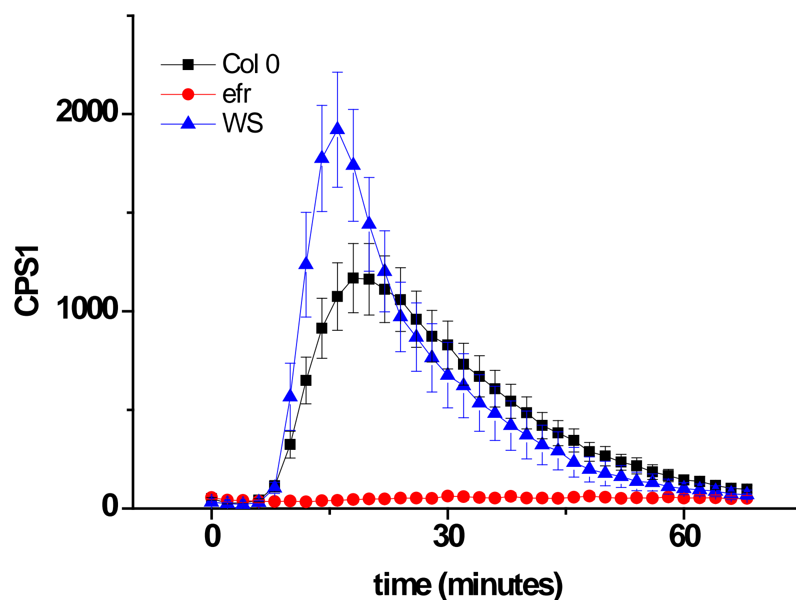
another microbial MAMP the elongation factor Tu (EF-TU) activates signaling events and defense responses that are common to flg22 (Zipfel et al., 2006). The *Arabidopsis efr* mutant lacks the functional EFR receptor and is unable to recognize the elicitor elf18.

Taking advantage of WS-0 insensitivity to flg22 and *efr* mutants' non responsiveness to elf18, we conducted baseline experiments to confirm the proper functionality of our experimental system. As evident from Fig6 both flg22 and elf18 induced a transient oxidative burst in the wild type Col-0 plants.

However, neither MAMP could elicit a response in their respective receptor receptor mutant plants. On the other hand a clear cut response could be seen in *efr* and WS-0 ecotypes when challenged by flg22 and elf18, respectively. These findings are indicative of the functionality of the system being used for conducting experiments with *Azospirillum*.

3.1.2.2 *Azospirillum* Sp7 induces an oxidative burst in Col-0 plants

In an attempt to compare the early responses exhibited by *Arabidopsis* plants upon recognition of PAMPs (like flg22 and elf18) and the elicitor from the PGPR *Azospirillum brasilense* Sp7, we exposed Col-0 leaf discs to Sp7 lysates**.** Thereby we found that Sp7, like flg22 and elf18, transiently induced the generation of reactive oxygen species (H_2O_2) in the wild type Col-0 plants (Figure 7). These results revealed a possible role of ROS in the *Azospirillum-Arabidopsis* interaction and in the post recognition responses manifested by the plant. Besides, it also highlights the similarities in the responses by the plant after recognition of a pathogenic and growth promoting bacteria.



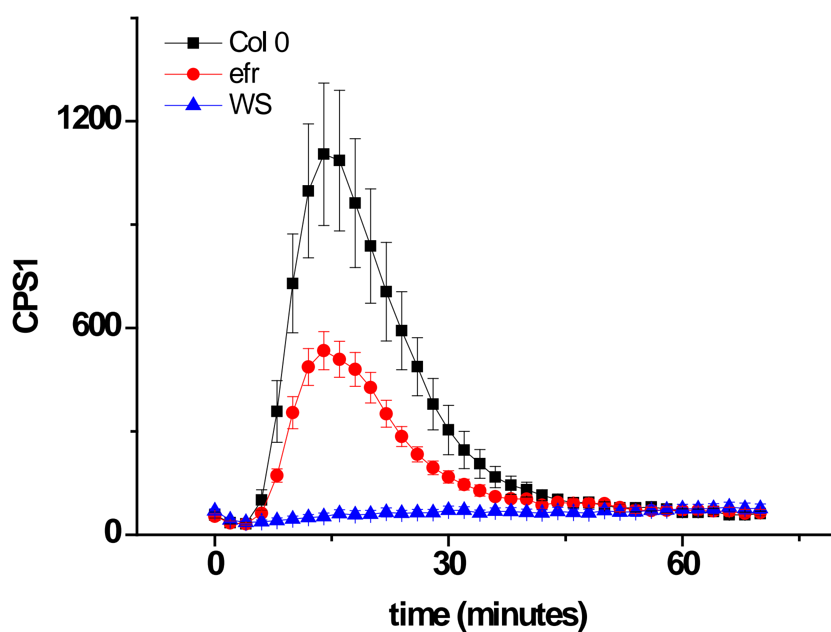


Figure 6 **H_2O_2 production by different ecotypes and *efr* mutant of *Arabidopsis*** when challenged with 500nM each of elf18 (above) and flg22 (below). Data represent mean values of n = 8 ± SE

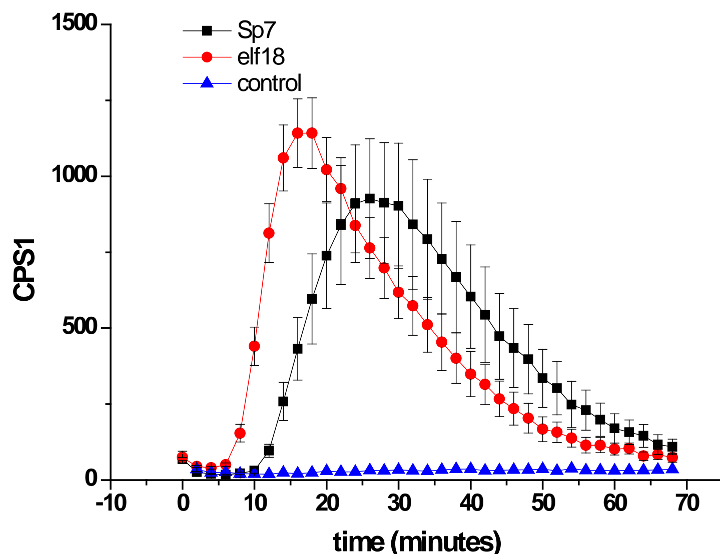


Figure 7 **ROS production by *A.brasilense* (100µg/ml) lysates and elf18 in wild type Col-0 plants.** The negative control represents the reaction mix with hepes buffer instead of an elicitor. Data represent mean values of n = 8 ± SE

3.1.2.3 The *Arabidopsis efr* mutant are insensitive to Sp7 lysates

Once established that ROS production has a role in the initial recognition events in the mutualistic interaction, we were interested to know whether or not the WS-0 ecotype and *efr* mutants respond to Sp7 in the manner observed for the wild type *Arabidopsis* plants. The choice of these mutants was basically the continuation of the results obtained in the pH experiments, which had shown that the pH response observed in suspension-cultured cells in response to *Azospirillum brasilense* is based on a proteinaceous elicitor and interfered with elf18 signaling. Leaf discs of the above mentioned mutants when exposed to *A.brasilense* lysates responded differently as depicted in Fig.8.

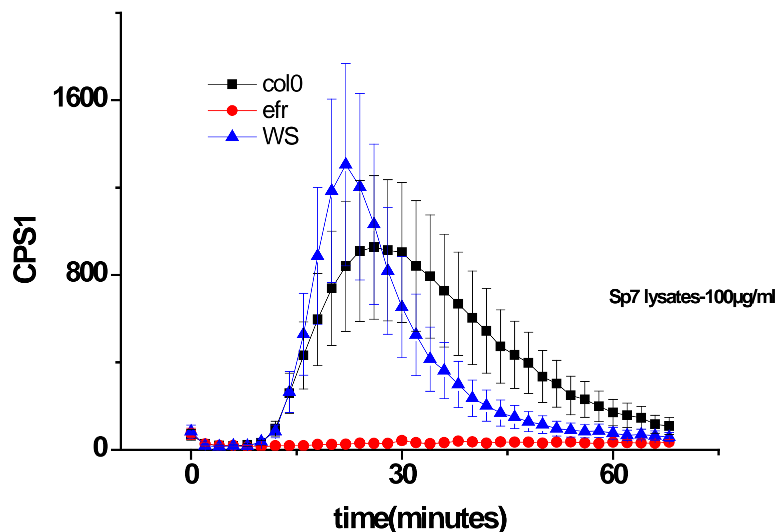


Figure 8 **Response of *efr* and WS-0 leaf discs to *Azospirillum* lysates**; H_2O_2 production upon exposure to 100µg/ml Sp7 lysates. Data represent mean values of n = 8 ± SE

While the *efr* mutant did not produce any H_2O_2 upon lysates application the WS-0 plants behaved in wild type manner. Non responsiveness of the *efr* mutants are predictive of a role of the EFR receptor-like kinase in the *Azospirillum* interaction with *Arabidopsis*. At the same time these experiments exclude a role of FLS2 in recognition or in the responses thereafter incurred by *Azospirillum* Sp7. It may also be noted that both elf18 and Sp7 lysates elicited quantitatively higher responses in WS-0 plants compared to Col-0.

3.2 Transcriptome profiling

In an attempt to identify statistically significant and reproducible changes in gene expression of *Arabidopsis thaliana* over the time-course of *Azospirillum* sp. infection, we assayed two weeks old mock inoculated and *Azospirillum* inoculated seedlings samples for four different time points (fig 9).

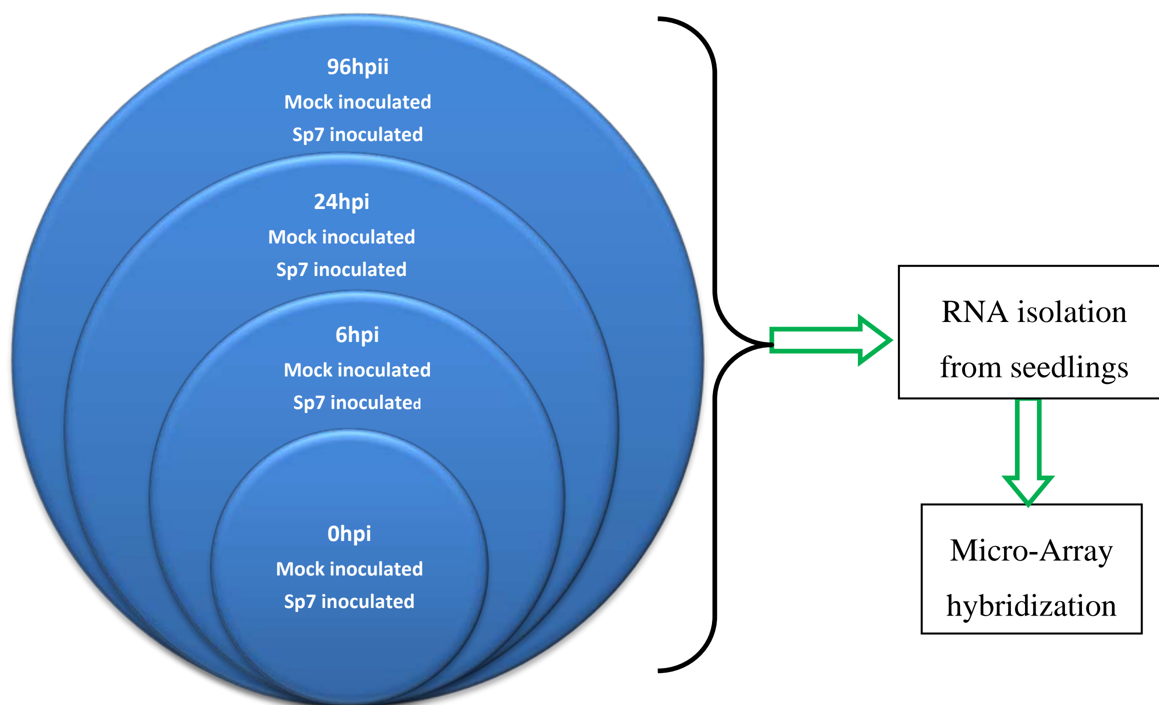


Figure 9 **Experimental designs for microarray analysis.** Three biological replicates were used each for mock and Sp7 inoculation.

3.2.1 Genetic responses-6 hours post inoculation (6hpi)

Transcriptome profiling of *Arabidopsis* seedlings following a 6h Sp7 treatment revealed the differential expression of only a few genes. The analysis of the array data showed a total of 35 genes exhibiting altered expression between bacterium inoculated and mock inoculated plants. Twenty five (25) of these transcripts were found induced while the rest showed repressed expression levels.

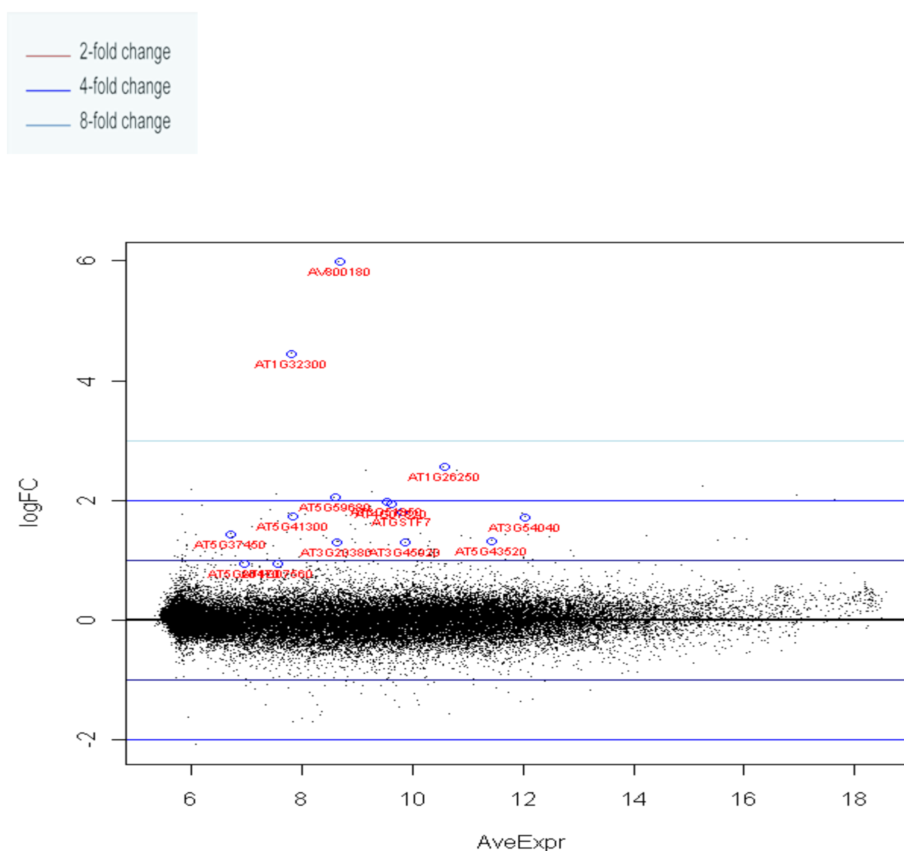


Figure 10 Scatter plot showing the average expression values (x-axis) of *Arabidopsis* genes and changes in log2 scales (y-axis) upon treatment with *Azospirillum brasilense* SP7**.**

In order to functionally categorize the regulated genes in the microarray data and to further know the over or under representation of these classes, we took advantage of MAPMAN, a widely used microarray analysis tool (Thimm et al., 2004). The MAPMAN microarray data analysis tool puts thousands of *Arabidopsis* genes into a set of hierarchical functional categories (bins, sub bins….individual enzyme). The first and more general tier of this particular division is called bin which encompasses, among others, categories like signaling, stress, secondary metabolism, DNA, RNA, protein etc. In 6hpi samples, MAPMAN based classification highlighted different pathways affected by the PGPR infection. The below given pie chart depicts share of the different pathways:

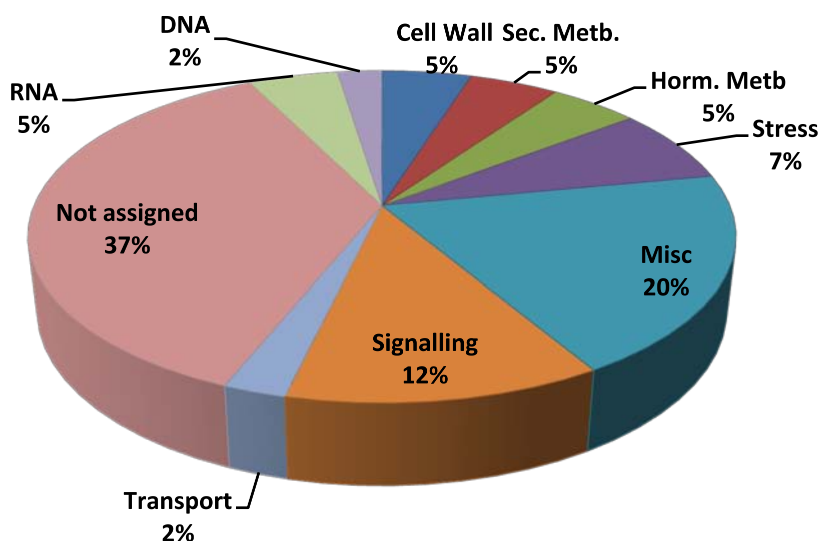


Figure 11 **Gene regulation trend.** Highly represented pathways with percentage of differentially regulated genes 6hpi.

3.2.1.1 Signaling

The proteins functioning in signaling pathways and stress were the most highly affected ones preceded by genes of unknown function (i.e. not assigned) and miscellaneous classes upon treatment with the bacteria. Members of all five receptor kinases in the signaling class were induced. These receptor like kinases are not fully characterized, however, it is expected that they are somehow involved in the initial interaction of plant with the bacteria in question. Besides this, another putative CHP-rich zinc finger protein, (at2g42060) predicted to be involved in intracellular signaling manifested increased expressions (1.60 LFC) in the treated samples.

3.2.1.2 Defence

The response of *Arabidopsis* plants to *Azospirillum* in terms of mounting defense related genes appeared not very drastic. Only two putative PR proteins are up regulated along with at3g13610 which is involved in scopoletin formation in *A. thaliana* (Kai et al., 2008). Scopoletin, a coumarin, originates from the general phenylpropanoid pathway with established role in plant defenses. Besides, decreased expression levels of two salicylic acid related proteins were observed.

It is interesting to note here the regulation pattern of at least three genes falling in not assigned, miscellaneous and secondary metabolism classes and functioning in terpenoid metabolism, which were repressed as a result of inoculation. Two of these proteins (at5g48010 & at5g48000) are part of a four member gene cluster. At5g48010 is shown to be required for synthesis of thalianol in *Arabidopsis* roots while at5g48000 in the breakdown of thalianol to the downstream products (Field and Osbourn, 2008). The same authors also predicted thalianol as detrimental to plant growth which reveals an indirect mechanism *Azospirillum* might resort to ensure growth promotion of *Arabidopsis*. At1g02920 (AtGSTF7), a Glutathione *S*-transferase of the phi class, is predicted to play a role in bacterial defense and salt stress. Its induction after 6hpi is an indicative of another pathway employed by Sp7 treatment to defend plants against pathogenic bacteria.

3.2.1.3 Others

In addition to the above mentioned genes mainly working in perception and defense mechanisms of plants, there were more which were mostly uncharacterized. We came across to a few up regulated genes having FAD binding domains with suggested oxidoreductase activity. Among the relatively better investigated genes, at4g28850 is predicted to be involved in strengthening the side-walls of root-hairs and cell walls in the root differentiation zone after the completion of cell expansion. (Maris et al., 2009). The induction of this protein might be related to mechanism (s) adopted in the *Azospirillum-Arabidopsis* interaction leading to altered root architecture.

A cytochrome P450, family protein (CYP81F2) attained higher transcripts upon interaction with Sp7. It is suggested to be part of the Indole glucosinolates biosynthesis pathway. Furthermore, an ABC transporter family protein (at3g28345) having ATPase activity coupled to transmembrane movement of unknown substrates was found to be with reduced transcripts (-1.36 LFC). This protein is also the one exhibiting the highest down regulation in terms of fold change in this particular time point followed by two SA related genes. At1g32300, a FAD domain containing protein, on the other hand emerged as

the highest induced protein succeeded by at1g26250, a cell wall related putative proline-rich extensions.

MAPMAN bin	Gene	Annotation	LFC
Cell wall	At1g26250	Proline-rich extensin. putative	2.52
	At4g28850	Xyloglucan transferase. putative	1.35
Secondary metabolism	At4g13300	Terpenoid synthase 13	-1.04
Miscellaneous	At5g48000	cytochrome P450 monooxygenase (AtCYP708A2)	-1.04
	At1g17180	GST- glutathione transferase activity(tau)	-1.31
	At1g02920	GST- glutathione transferase activity(phi)	1.82
	At5g57220	CYP81F2 (cytochrome P450. family 81	1.90
	At1g30730	FAD-binding domain-containing protein	1.07
	At1g32300	FAD-binding domain-containing protein	4.4
	At1g26380	FAD-binding domain-containing protein	1.96
	At1g26410	FAD-binding domain-containing protein	1.37
Hormones	At5g38020	SA synthesis degradation	-1.35
	At5g37970	SA synthesis degradation	-1.35
Stress	At4g07820	PR protein. putative similar to PR1	1.86
	At4g14630	Germin-like protein	2.09
	At4g14060	MLP-related	-1.09
Signalling	At3g54040	Photoassimilate-responsive protein-related	1.92
	At5g59680	Leucine-rich repeat protein kinase. putative	2.15
	At5g37450	Leucine-rich repeat protein kinase. putative	1.38
	At3g45920	receptor protein kinase-related	1.37
	At5g41300	receptor-like protein kinase-related	1.61
Transport	At3g28345	ABC transporter family protein	-1.41
Not assigned	At5g48010	THALIANOL SYNTHASE 1	-1.08

Table 13 Differentially regulated genes 6hpi with log2 fold ratios

3.2.2 Genetic responses-24 hours post inoculation (24hpi)

The genetic responses in terms of number of genes increased at 24hpi time course. We identified sixty three (63) genes showing altered expression levels in the *Azospirillum* challenged plants. The induced and repressed genes numbered thirty three (33) and thirty (30) respectively. Genes pertaining to hormone metabolism and cell wall MAPMAN bins appeared as the most affected after inoculation followed by “miscellaneous” and “not assigned” classes. Both of these bins contained equal numbers of differentially regulated genes. Treatment with Sp7 also altered the expression of genes belonging to

different MAPMAN classes like stress, signaling and RNA.

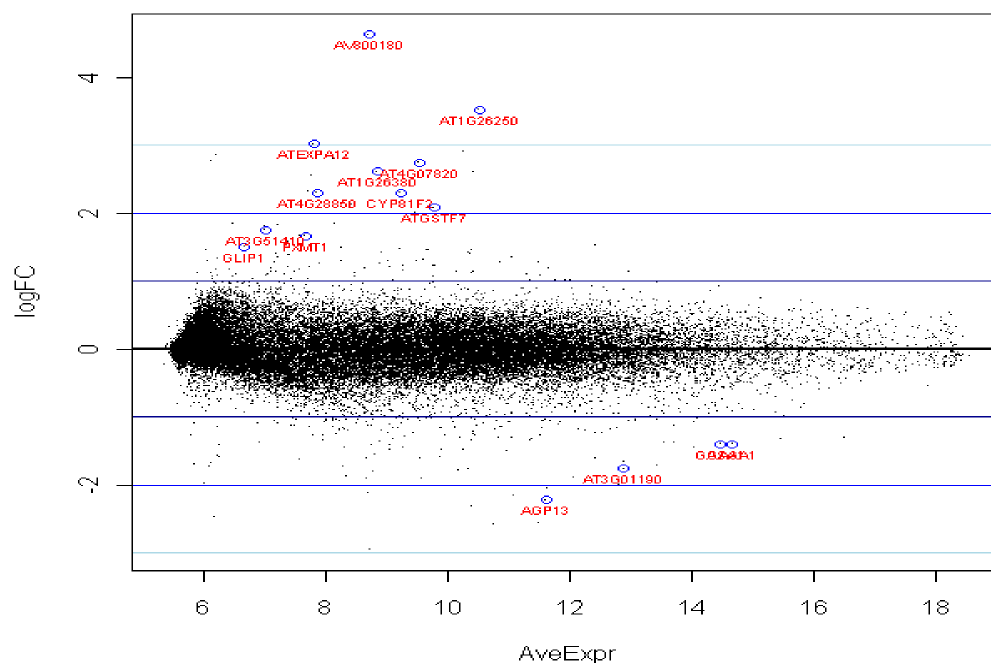


Figure 12 **Scatter plot** showing the average expression values (x-axis) of genes and changes in log2 scales (y-axis) upon treatment with *Azospirillum brasilense* Sp7

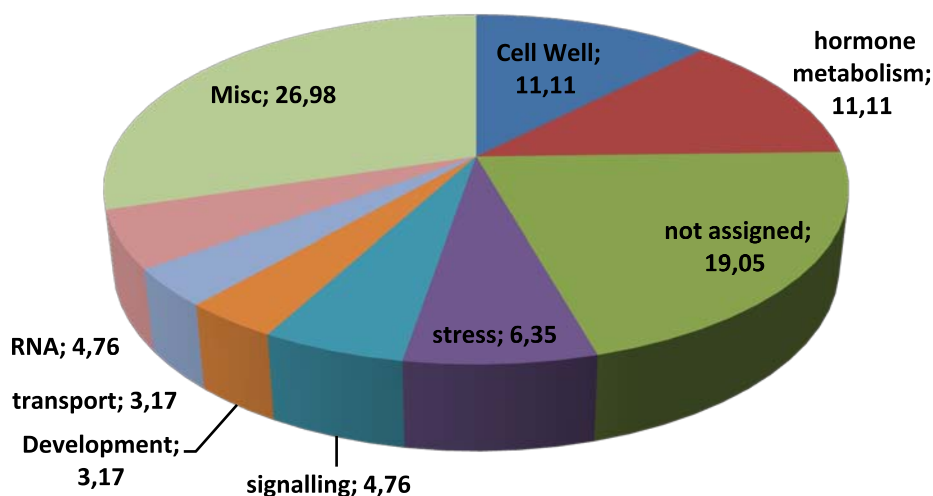


Figure 13 **Gene regulation trend** - Highly represented pathways with percent of differentially regulated genes 24hpi

3.2.2.1 Lipid transfer proteins (LTPs)

The class of proteins most distinctly and specifically expressed with altered levels after 24h of inoculation was related to the protease inhibition/lipid transfer proteins (LTPs) sub class. LTPs which are small, cationic, cysteine-rich peptides are connected with a variety of functions including cutin biosynthesis, defense signaling, production of anti microbial compounds and calmodulin-binding. Because of their responsiveness to pathogens, they have been classified as member of the pathogenesis proteins and placed in PR-13 group. All six of proteins with differential regulation in our array data have been predicted to be part of the lipid transfer/lipid binding process. Noteworthy is their down regulation trend without any exception. Although a variety of functions have been proposed for different LTPs the transcripts relating to the same class and showing regulation as a result of *Azospirillum* treatment are not functionally characterized.

MAPMAN bin	Gene	Annotation	LFC
Miscellaneous	At1g62510	protease inhibitor/seed storage/ LTP	-1.28
	At4g12490	protease inhibitor/seed storage/ LTP	-2.64
	At4g12510	protease inhibitor/seed storage/ LTP	-2.07
	At5g46900	protease inhibitor/seed storage/ LTP	-1.96
	At4g12520	protease inhibitor/seed storage/ LTP	-1.61
	At4g22460	protease inhibitor/seed storage/ LTP	-1.42
	At5g46890	protease inhibitor/seed storage/ LTP	-2.61

Table14 Differentially regulated LTP genes 24hpi with log2 fold ratios

3.2.2.2 Cell wall

We found two cell wall related genes at1g26250 & at4g28850 exhibiting common up regulation not only in 24 hpi but also for the earlier 6hpi time course. At1g26250 a cell wall related putative proline-rich extension which was among the two highly induced proteins at the 6hpi time point, showing even higher transcript accumulation in 24hpi. The same pattern was also seen for, a putative xyloglucan: xyloglucosyl transferase (at4g28850). Both of these genes function in modification of the cell wall hinting the way PGPR/plant interaction affect the plant cell wall. In addition to the mentioned genes, another root

specific cell wall structural constituent proline rich protein, at1g54970, was up regulated with the PGPR colonization. At least three genes in this class came up with decreased expressions after treatment with Sp7. Although not fully characterized, one member of the repressed genes, at1g05660, which is a putative polygalacturonase/pectinase, is suggested to play a role in carbohydrate metabolic process while another, a pectinesterase family protein (at2g45220) is putatively involved in cell wall modifications.

MAPMAN bin	Gene	Annotation	LFC
Cell wall	At4g26320	AGP13 (ARABINOGALACTAN PROTEIN 13)	-2.23
	At1g54970	ATPRP1 (PROLINE-RICH PROTEIN 1)	1.38
	At1g26250	proline-rich extensin. putative	3.44
	At1g05660	Polygalacturonase putative / pectinase. putative	-1.33
	At3g15370	ATEXPA12 (*ARABIDOPSIS THALIANA* EXPANSIN 12)	2.74
	At4g28850	xyloglucan:xyloglucosyl transferase. putative	2.29
	At2g45220	pectinesterase family protein	-1.26

Table 15 Differentially regulated genes in the cell wall bin, 24hpi with log2 fold ratios.

3.2.2.3 Hormone metabolism

We encountered a new set of proteins as compared to the 6hpi where only two SA related genes were repressed by inoculation. Four auxin responsive genes of unknown function were differentially regulated pointing towards auxin participation in *Arabidopsis*/*Azospirillum* interaction. Similarly one clone pertaining to ethylene biosynthesis was up regulated. Besides, ethylene biosynthesis, at3G49700 alias 1-aminocyclopropane-1-carboxylate synthase (AtACS9) is also shown to be induced during hypoxia. The enhanced expression in our case, however, vanished before 96hours.

We could notice the down regulation of a gibberellic acid (GA) responsive gene in our array. Based on the known annotation, the repressed protein in addition to be responsive to gibberellins was also responsive to brassinosteroid (BR)

and abscisic acid stimuli. This regulation was however antagonistic by BR and GA.

MAPMAN bin	Gene	Annotation	LFC
Hormone metabolism	At2g33830	dormancy/auxin associated family protein	-1.31
	At5g20820	auxin-responsive protein-related	1.21
	At3g15450	similar to unknown protein-96	-1.37
	At5g10990	auxin-responsive family protein	1.02
	At3g49700	ETO3 (ETHYLENE OVERPRODUCING 3)	1.87
	At1g75750	GASA1 (GAST1 PROTEIN HOMOLOG 1)	-1.30
	At3g16390	Encodes a nitrile-specifier protein NSP3	-1.65

Table 16 Differentially regulated genes 24hpi related to hormone metabolism with log2 fold ratios

3.2.2.4 Stress / defense

A putative PR1 like protein along with a Glutathione *S*-transferase of the phi class (AtGSTF7), continued with its augmented transcripts even at 24 hours post inoculation signifying that their role is not limited only to the initial interaction processes between the Sp7 and Col.0. Another transcript annotated as disease resistance-responsive protein-related / dirigent protein-related putatively engaged in lignin biosynthesis was down regulated. .

Some cytochrome P450 monooxygenase family proteins were modified upon treatment with the PGPR. CYP81F2, -participating in Indole glucosinolates biosynthesis- maintained its induction. In addition, we observed another member of the cytochrome P450 family, AtCYP71A12 with amplified expression. This protein is not only responsive to bacteria but is at the same time predicted to be part of camalexin synthesis together with AtCYP71A13, which contributes a major part in this process.

A secreted lipase with a GDSL-like motif designated GDSL LIPASE1 (GLIP1) has been shown to directly interfere with fungal infection processes by acting on fungal cell walls through its action as an antimicrobial compound. Furthermore, it is a vital constituent for both local and systemic resistance responses in the incompatible interaction with *Alternaria brassicicola*. These interactions have been shown to be dependent on ethylene signaling pathway. GLIP1 levels

increased significantly upon Sp7 treatment. This might correspond to importance of ethylene signaling in mounting antifungal defenses in PGPR interaction with *Arabidopsis*.

MAPMAN bin	Gene	Annotation	LFC
Stress	At4g07820	Pathogenesis-related protein.	2.70
	At2g39430	Disease resistance protein-related	-1.37
	At1g80920	J8; heat shock protein binding	-1.08
	At4g14630	GLP9- Germin-like protein	1.59
	AT5G40990	GLIP1- GDSL LIPASE1	1.43
Misc.	At1g02920	AtGSTF7	1.82
	At5g57220	CYP81F2 (cytochrome P450. family 81)	2.30
	At2g30750	CYP71A12 (cytochrome P450. family 71)	2.43

Table 17 Differentially regulated genes related to stress and miscellaneous classes, 24hpi with log2 fold ratios

3.2.2.5 Others

We observed decreased transcripts for a putative peroxidase (at4g30170) and peroxidase27 (at3g01190) after inoculation. These are suggested to be involved in response to oxidative stress and are known to exhibit heme binding. A basic helix loop helix and a MYB 15 transcription factors were respectively repressed and induced after 24 hours of inoculation. While no known function is documented for the former, the later is shown to respond to ABA and improved tolerance to drought and salt stresses. As the other ABA responsive genes remained unaffected in our data, the induction of MYB15 seems to be ABA independent here. The NAC-type transcription factor (AtANAC042) supposedly working in multicellular organismal development processes was another induced gene. Decreased expression levels surfaced after inoculation for a tonoplastic intrinsic proteins/water channels TIP2.3 (at5g47450) and another aquaporin NOD26-like intrinsic protein NLM1.1 (at4g19030). The latter has been shown to be responsible for arsenite transport into roots of *Arabidopsis* (Kamiya et al., 2009).

3.2.3 Genetic responses-96 hours post inoculation (96hpi)

Contrary to the early time points, a tremendous hike in the number of differentially expressed gene was noticed after 96 hours of inoculation. Of 1174 genes with modified expression levels, 724 manifested at least two fold higher transcripts after being treated with *Azospirillum* while the remaining 450 genes exhibited retarded expressions as compared to non-inoculated plants. Owing to these encouraging results, we carried out a detailed analysis, primarily using the fore used array analysis tool MAPMAN.

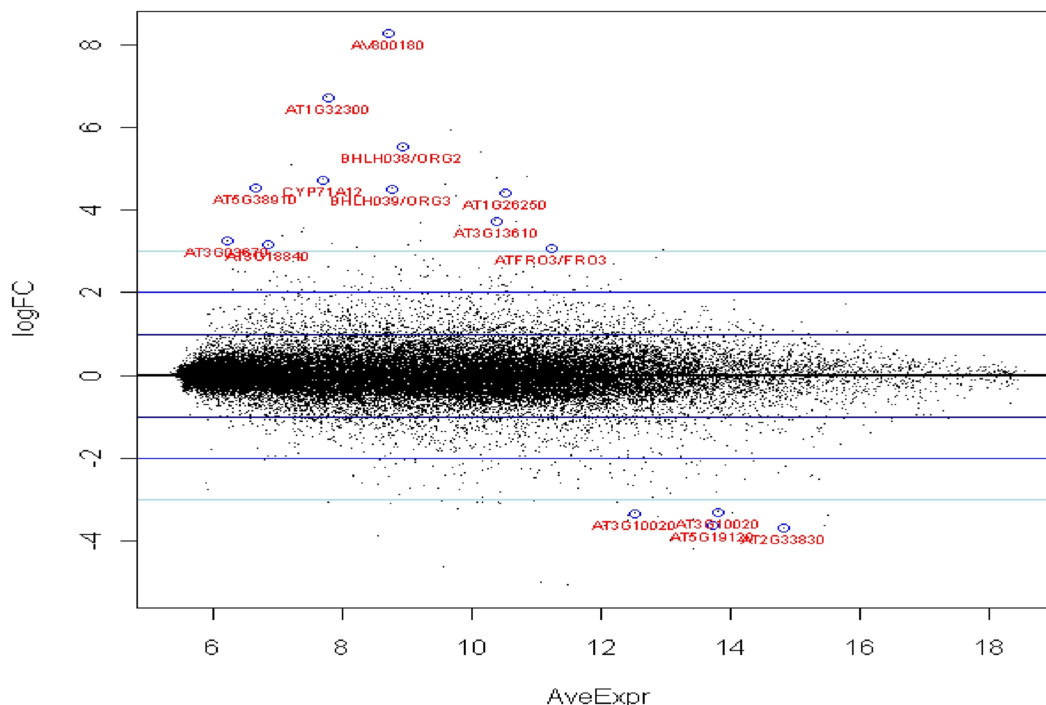


Figure 14 Scatter plot showing the average expression values (x-axis) of genes and changes in log2 scales (y-axis) 96hpi with *Azospirillum brasilense* Sp7.

Prior to functional classification, we tried to trace the localization of genes in different cellular compartments. Using Gene Ontology (GO), cellular compartments for *Arabidopsis* whole genome were determined first followed by determination of 96hpi differentially regulated genes. A significant increase in the activity of genes positioned either in cell wall or extracellular regions after

treatment with Sp7 was observed. Conversely a decrease was seen in endoplasmic reticulum and golgi apparatus localized proteins (Fig 15). Based on these observations, the PGPR in question probably modulate the cell wall and extra cellular proteins to a greater extent in the later time point.

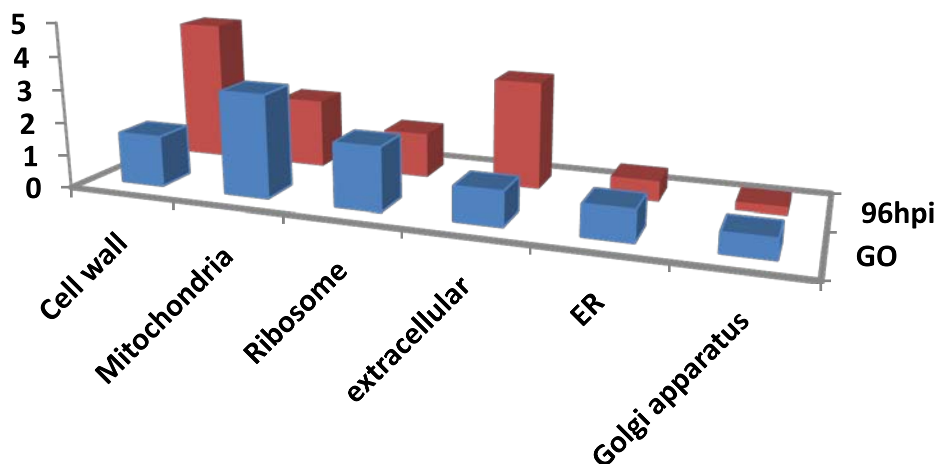


Figure 15 **Gene localization pattern (96hpi) using GO as background.** The y axis represent percentages of the total genes for GO and percent of differentially regulated genes for 96hpi.

In order to abreast ourselves with the trend of gene expression behavior in the late time point upon Sp7 inoculation, we initially resorted to a broader classification of our data into bins. The pie chart in Fig 16 underlines the most highly representative eleven classes in 96hpi data. Similar to findings in the early time points, most of the genes with modified expression belonged to “not assigned” and “miscellaneous” bins. Together the two contributed more than 40 % of the total number. The other prominent broad categories were RNA, protein, stress, cell wall, development and others.

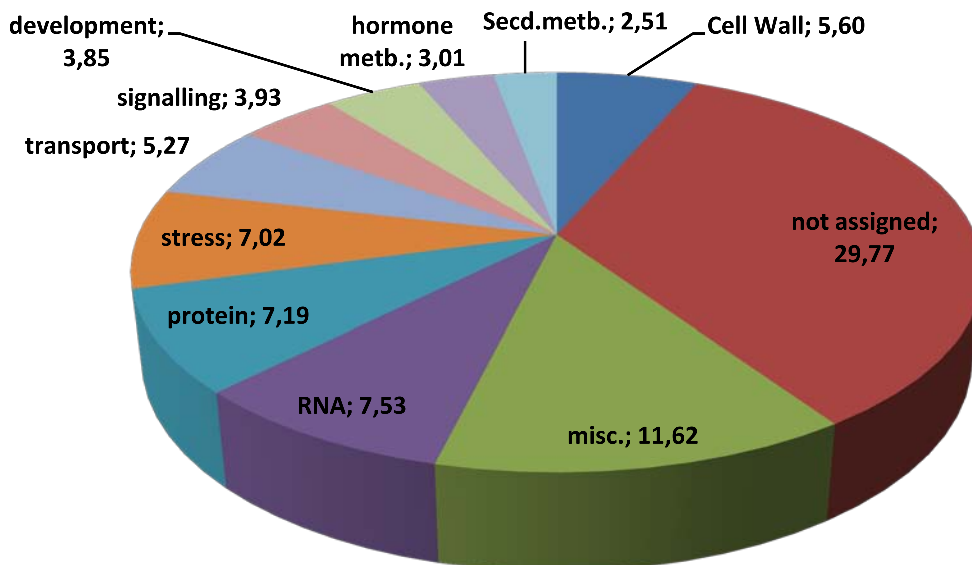
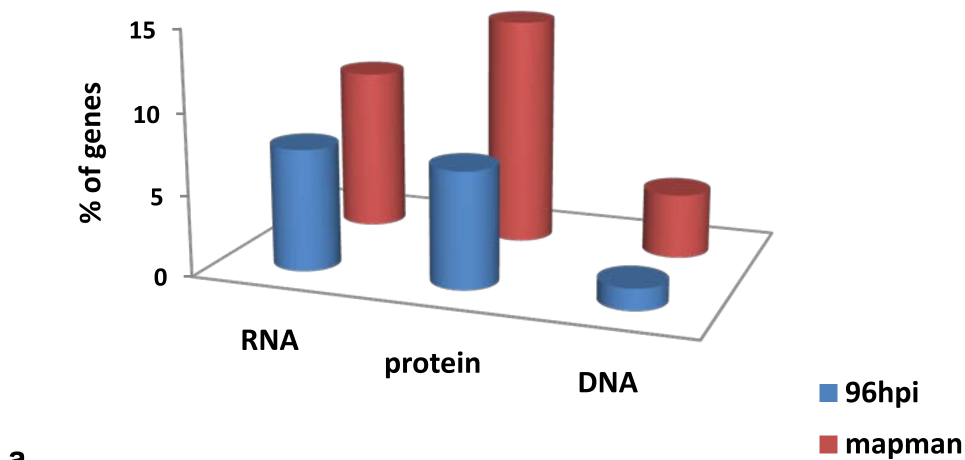
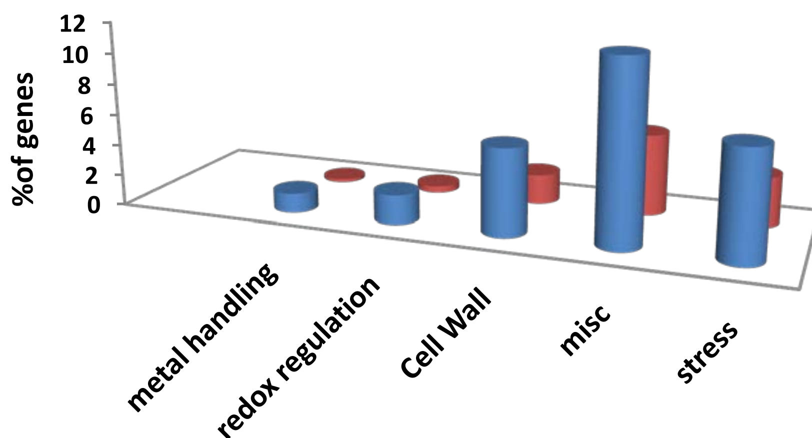


Figure 16 **Gene regulation trend** - Highly represented pathways with percent of differentially regulated genes 96hpi

It should, however, be noted that each MAPMAN bin contains different numbers of genes for that particular class. When the analysis takes into account the ratio of differentially regulated genes in a specific MAPMAN bin to the total number of genes in that bin, the representative order of genes is modified to some extent. In other words the use of MAPMAN as background results in under representation of few classes which looked over representative in the earlier analysis (Fig 17a). These included RNA, protein and DNA bins. However the categories like stress, misc. and cell wall continued to be well represented even in the new scenario (Fig 17b).



a



b

Figure 17 **Gene regulation trend using MAPMAN as background**. **a)** under represented pathways **b)** over represented pathways- 96hpi

The representative bins broadly guided us to the trend of gene expression upon Sp7 inoculation. However, taking advantage of the hierarchical division of functional groups in the MAPMAN tool, we turned towards narrowing down our search to find out a more specific trend of gene regulation after treatment with *Azospirillum*. The sub bins revealed thereby are summarized in the given table below:

Bin	Sub bin	Regulated genes	Up regulated	Down regulated
Cell wall		63	55	8
	Cell wall.modification	21	19	2
	Cell wall proteins	16	16	0
Secondary metabolism		32	25	7
	Flavonoids	14	11	3
	Isoprenoids	7	5	2
Hormone metabolism		36	19	17
	Ethylene	11	3	8
	Auxin	10	6	4
Stress		82	60	22
	Biotic	45	35	10
	A-biotic	37	25	12
Redox.regulation		22	7	15
	Redox.thioredoxin	6	1	5
	Redox.glutaredoxins	9	3	6
Misc.		140	103	37
	Cytochrome P450	25	15	10
	Peroxidases	19	15	4
	UDP glucosyl and glucoronyl transferases	14	7	7
	Gluco-, galacto- and mannosidases	13	10	3
	Glutathione S transferases	12	12	0
RNA		88	47	41
	Regulation of transcription	82	41	41
DNA		16	13	3
	Synthesis/chromatin structure	12	10	2
Protein		88	43	45
	Protein.degradation	49	14	35
	postranslational modification	18	14	4
Signalling		46		
	Signalling.receptor kinases	20	16	4
	Signalling.calcium	12	9	3

Table 18 Number of differentially regulated genes 96hpi in respect of highly representative MAPMAN bins and sub bins

3.2.3.1 Cell wall

Among many sub classes of the cell wall category in the array analysis tool, the two most prominent sub groups in our data were cell wall modification and cell wall proteins. For both of the stated sub classes mostly these genes were up regulated. Only two transcripts engaged in cell wall modification while none for cell wall proteins was down regulated.

Cell wall modification

Concerning cell wall modification proteins, they were either expansins or xyloglucanases. The expansins, in general are responsible for uni dimensional cell growth and cell wall loosening & organization. Xyloglucanases, on the other hand, are chiefly engaged in glucan metabolic processes. Out of 11 differentially expressed expansins 9 were members of the□-expansins (EXPA) sub gene family. Of these nine proteins only one exhibited reduced levels of expression upon inoculation while the rest showed accumulated transcripts.

Although availability of information on the specific functions of single members of the expansin gene family is scarce, some genes have been investigated in more detail. AtEXPA7 and AtEXPA18, for instance, are root specific expansins whose expression is tightly linked to root hair initiation (Cho and Cosgrove, 2002). Enhanced expression in our results might indicate them as one of the potential actors in the way *Azospirillum* affect the *Arabidopsis* root system.

Two more expansins manifested differential regulation in the treated samples. One of them is member of the ß-expansins (EXPB) gene family while the other of the expansin-like family B (EXPLB). The EXPB family proteins are thought to act in the similar wall loosening functions as EXPA. However, functioning of EXPLB is still unascertained.

The other prominent class of proteins within cell wall modification group was xyloglucanases. There were 10 xyloglucanases showing modified expression following inoculation. Nine proteins arose with accumulated transcripts and only one behaved inversely. In addition to be part of glucan metabolism, some xyloglucanases appear to function in defense and developmental processes.

For example XTH33 (at1g10550) a xyloglucan endotransglucosylase-hydrolase (AtXTH33), has a role in protecting plant against aphids (Divol et al., 2007). In addition, AtXTH33 is co-regulated with Peroxidases and the anion transporter SLAH3 suggesting a role for these proteins in the interaction process (see discussion). Primary root elongation, on the other hand, was reported to be connected to the expression of another xyloglucan XTH18 (at4g30280) (Osato et al., 2006), which is directly co-regulated with AtXTH33. Besides, two further members of the Arabidopsis XTH family (at4g28850 & at4g25820) having roles in strengthening the side-walls of root-hairs and cell walls in the root differentiation zone after the completion of cell expansion were seen with increased expression levels. (Maris et al., 2009).Interestingly the former xyloglucan is regulated in upward manner in all time points.

AGI	Gene name	LFC
At1g69530	ATEXPA1- (*ARABIDOPSIS THALIANA* EXPANSIN A1)	-1.23
At3g29030	EXPA5-(*ARABIDOPSIS THALIANA* EXPANSIN A5	1.34
At1g12560	ATEXPA7-(*ARABIDOPSIS THALIANA* EXPANSIN A7)	1.94
At5g02260	ATEXPA9- (*ARABIDOPSIS THALIANA* EXPANSIN A 9)-	1.15
At3g15370	ATEXPA12-(*ARABIDOPSIS THALIANA* EXPANSIN A 12)	2.70
At5g56320	ATEXPA14-(*ARABIDOPSIS THALIANA* EXPANSIN A14)	1.52
At2g03090	ATEXPA15-(*ARABIDOPSIS THALIANA* EXPANSIN A 15)	1.81
At4g01630	ATEXPA17- (*ARABIDOPSIS THALIANA* EXPANSIN A17)	1.18
At1g62980	ATEXPA18-(*ARABIDOPSIS THALIANA* EXPANSIN A18)	1.98
At4g17030	ATEXLB1 (At. EXPANSIN-LIKE B1	1.39
At4g28250	ATEXPB3	1.45
At4g28850	Xyloglucan-xyloglucosyl.transferase..putative	3.45
At1g11545	xyloglucan:xyloglucosyl transferase. putative	1.50
At5g57530	xyloglucan:xyloglucosyl transferase. putative	2.07
At4g30280	XTH18 (XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 18)	1.17
At5g48070	XTH20 (XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 20)	2.21
At1g10550	XTH33- Xyloglucan-xyloglucosyl.transferase/hydrolase 33	1.26
At3g44990	XTR8- 7 (XYLOGLUCAN.ENDOTRANSGLYCOSYLASE.7)	1.53
At4g25820	XTR9 (XYLOGLUCAN ENDOTRANSGLYCOSYLASE 9); hydrolase	2.59
At2g01850	EXGT-A3 – endo-xyloglucan.transferase.A3) .hydrolase	-1.17
At5g13870	EXGT-A4 – endo-xyloglucan.transferase.A4);.hydrolase	1.41

Table 19 Differentially regulated genes related to cell wall modification with log2 fold ratios.

Cell wall proteins

The cell wall proteins are believed to be involved in various plant growth and developmental aspects. The wall proteins significantly modified upon inoculation with Sp7 in our case were dominated by arabinogalactan and proline rich proteins. Interestingly all sixteen genes in this sub bin were up regulated**.**

Four AGPs and one FASCICLIN-LIKE arabinogalactan 6 (FLA6) exhibited higher transcript accumulation in our array data. Most of AGPs still await functional characterization but among the better studied genes, AGP1 and AGP2 are reported to be responsive to Aluminum stress. Besides, AGP2 is up regulated when exposed to infection with *X.campestri.* This induction was partly ethylene dependent (Schultz et al., 2002). Its induction by *Azospirillum* reflects its responsiveness to wider array of biotic stresses.

The proline rich proteins (PRPs) and proline rich extensin like family proteins are structural constituents of the cell wall, predicted to be involved in plant cell wall organization. We found six such proteins to be up regulated upon Sp7 inoculation. The AtPRP3 is one of the better studied proteins among them. Its expression has been shown to be controlled by regulatory pathways specific for root hair development thus underscoring its contribution to cell wall structure in *Arabidopsis* root-hair bearing epidermal cells (Bernhardt and Tierney, 2000).

AGI	Gene name	LFC
At5g64310	AGP1 (ARABINOGALACTAN PROTEIN 1)	1.38
At2g22470	AGP2 (ARABINOGALACTAN PROTEIN 2)	1.42
At4g40090	AGP3 (arabinogalactan-protein 3)	2.20
At4g37450	AGP18 (ARABINOGALACTAN PROTEIN 18)	1.39
At5g40730	AGP24(ARABINOGALACTAN PROTEIN 24)	1.48
At2g20520	FLA6 (FASCICLIN-LIKE ARABINOGALACTAN 6)	2.87
At1g54970	ATPRP1 (PROLINE-RICH PROTEIN 1)	2.55
At3g62680	PRP3 (PROLINE-RICH PROTEIN 3)	2.04
At2g46630	Hypothetical protein	1.31
At1g26240	Proline-rich extensin-like family protein-	1.02
At1g26250	Proline -rich extensin. putative	4.88
At4g08400	Proline -rich extensin. putative	1.25
At4g08410	Proline -rich extensin. putative	1.55

Table 20 Differentially regulated cell wall protein sub bin genes with log2 fold ratios.

3.2.3.2 Secondary metabolism

Azospirillum brasilense caused significant changes in expression patterns of *Arabidopsis* proteins functioning in secondary metabolism particularly flavonoids and isoprenoids. Together they constituted about 65% of the differentially regulated secondary metabolism related genes.

Flavonoids

Flavonoids are a class of secondary metabolites which are known to play an important role in the plant defense against oxidative stressors (Dakora and Phillips, 1996) like pathogens, herbivores, or abiotic factors. Besides, they exhibit a well established role in the legume–Rhizobium symbiotic interaction.

Fourteen (14) genes relating to flavonoid metabolism were found with altered expressions. Three of them (at3g29670, at5g39050 and at4g35420) displayed retarded transcript levels as compared to non-treated plants. The first two are transferase family proteins thought to be engaged in transferring acyl groups other than amino-acyl groups. The third down regulated protein DRL1 is a dihydroflavonol 4-reductase family proteinputatively involved in pollen and seed development.

AGI	Name	LFC
At5g13930	Chalcone synthase (AtCHS) - alias Transparent Testa 4 (TT4)	1,09
At5g05270	Chalcone isomerase (AtCHI)	1,88
At3g51240	Flavanone 3-hydroxylase (AtF3H) alias Transparent Testa 6	1,31
At5g42800	DIHYDROFLAVONOL 4-REDUCTASE (DFR)	1,95
At5g08640	flavonol synthase (AtFLS)	1,86
At4g22870	Anthocyanidin synthase (AtANS)	2,41
At4g22880	Leucoanthocyanidin dioxygenase (AtLDOX)	2,08
At1g03495	Anthocyanin 5-aromatic acyl-transferase (AtAAC)	1,48
At2g22930	UDP-dependent glycosyl transferase (AtUGT79B8)	1,88
At5g54060	UDP-dependent glycosyl transferase (AtUGT79B1)	2,02
At4g25310	Oxidoreductase,.2OG-Fe(II).oxygenase.family.protein.	1,18
At3g29670	Transferase.family.protein	-1,79
At5g39050	Transferase.family.protein	-1,05
At4g35420	dihydroflavonol 4-reductase family protein (DFR1)	-1,03

Table 21 List of the flavonoid related genes with log2 fold ratios

Eleven genes came about with increased expression after bacterium treatment. Many of them take part in the flavonoid biosynthesis pathway. As it is known that flavonoid biosynthesis proceeds from 4-coumaroyl-and malonyl-CoAs to naringenin chalcone. At5g13930 (CHS), a chalcone synthase, takes part in this step of the pathway. Chalcone is converted into the flavanone by chalcone isomerase (at5g05270-AtCHI). Both these CHS and CHI were showing a higher expression after exposure to *Azospirillum*. Flavanone is further hydroxylated by Flavanone 3_-hydroxylase (F3H or FHT) to form a dihydroflavonol (3-hydroxyXavanone or flavanonol). Again the enzyme F3H is induced in our studies. Subsequent reduction of DihydroXavonols to leucoanthocyanidins by dihydroXavonol 4-reductase (DFR) along the branch leading to flavan-3-ols, proanthocyanidins and anthocyanidins may take place. Alternatively, dihydroflavonol is oxidized to flavonol iand the reaction is catalysed by flavonol synthase (FLS). Enhanced expression of flavonoid biosynthesis pathway genes upon Sp7 inoculation is an indicative of the role of this molecule in plant *Azospirillum* interaction.

Still some additional functions for these proteins have also been documented. Chalcone synthase (CHS), for example, is reported to be involved in the regulation of auxin transport and the modulation of root gravitropism. AtF3H is predicted to be involved in protection of plants against UV-B irradiation (Li et al., 1993) . On the other hand, Leucoanthocyanidin dioxygenase (LDOX) is part of the proanthocyanidin biosynthetic pathways (Abrahams et al., 2003) in addition to its established role in the formation of anthocyanin (Nakajima et al., 2006).

It has been reported that AtMYBL2, is a negative regulator of the biosynthesis of anthocyanin in *Arabidopsis* (Matsui et al., 2008). Our findings are in complete agreement with the theme as can be observed on one hand by transcriptional repression of this particular TF and on the other by induction of the genes in the flavonoid biosynthetic process.

Terpenoids

Terpenes, a very large class of secondary metabolites, are well known for functioning in direct defense because of their ability to manifest toxic and

deterrent effects against plant pathogens and herbivores. Monoterpenes, however, are among terpenes which along with sesquiterpenes and a few diterpenes can volatize and thus can act as attractants or deterrents for pollinators and herbivores, or as signals released after damage is incurred by herbivores. In this way they are able to attract their predators and parasitoids. These volatiles are emitted not only from flowers and other aerial parts of the plant, but also from the roots. Release of these volatiles is often developmentally regulated or induced by damage.

AGI	Name	LFC
At3g14530	Putative geranylgeranyl pyrophosphate synthase (AtGGPS6)	1.69
At1g08550	NPQ1 (NON-PHOTOCHEMICAL QUENCHING 1)	-1.54
At3g25830	Putative terpene synthase	-1.03
At1g33750	Putative terpene synthase/cyclase family protein	1.38
At4g20230	Putative terpene synthase/cyclase family protein	1.28
At1g66960	putative lupeol synthase (AtLUP5)	1.21
At3g31415	Putative terpene synthase/cyclase family protein	1.46

Table 22 List of the terpenoid related genes with log2 fold ratios.

We observed only five genes with altered regulation patterns in the array results. Most of the transcripts are predominantly expressed in roots. The only protein expressing suppressed transcripts (at3g25830) had a terpene synthase-like sequence-1,8-cineole and was part of the monoterpenoid biosynthetic process. Among the remaining four which were induced, three proteins included putative terpene synthases (at1g33750, at4g20230 & at3g31415) which are thought to be involved in magnesium ion binding and possess lyase activity. AtLUP5 (at1g66960), a putative lupeol synthase, is also up regulated in the treated samples. Presumably it is involved in pentacyclic triterpenoid biosynthetic process. Notably Lupeol is only present in plants but not fungi and animals.

3.2.3.3 Hormone metabolism

The major portion of modified genetic responses in the treated plants in terms of hormone metabolism were mainly ethylene and auxin related. MAPMAN based

classification attributed 11 and 10 transcripts with altered ratios to ethylene and auxin sub classes respectively.

Ethylene

The differentially regulated genes in the ethylene metabolism section were predominantly down regulated after inoculation with Sp7. Only three of the eleven distinctly expressed genes showed enhanced expressions. The induced genes included at5g67430 which is a GCN5-related N-acetyltransferase (GNAT) family protein, at3g12900, a 2OG-Fe(II) oxygenase family protein supposedly having oxidoreductase activity and at2g19590 (ACO1) a 1-aminocyclopropane-1-carboxylate oxidase enzyme. The last of the three is comparatively better characterized which is part of the ethylene biosynthetic process. The enzyme ACC oxidase catalyses the last step of ethylene biosynthesis in plants, converting 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene.

AGI	Name	LFC
At5g67430	putative HLS1-like GNAT-type N-acetyltransferase	1.21
At1g03410	2A6; oxidoreductase	-1.03
At5g43450	putative iron-(II)-dependent dioxygenases (2-ODD).	-1.17
At5g43440	putative iron-(II)-dependent oxidoreductase	-1.74
At3g12900	putative iron-(II)-dependent oxidoreductase	3.01
At2g19590	ACO1 (ACC OXIDASE 1); 1-aminocyclopropane-1-carboxylate oxidase	1.39
At5g61600	ethylene-responsive element-binding family protein	-1.43
At5g51190	AP2 domain-containing transcription factor, putative	-1.14
At5g61590	AP2 domain-containing transcription factor family protein	-0.98
At3g11930	universal stress protein (USP) family protein	-1.45
At3g62550	universal stress protein (USP) family protein	-2.34

Table 23 List of the ethylene related genes with log2 fold ratios.

The down regulated transcripts in this category consisted of three (at1g03410, at5g43450 and at5g43440) putative iron-(II)-dependent oxidoreductases encoding proteins with sequence similarity to ACC oxidase and same number of ethylene responsive element factor (ERF). The ERF belonged to the subfamily B-3 of ERF/AP2 transcription factor family. It has been demonstrated that the overexpression of ETHYLENE RESPONSE FACTOR1 constitutively activated

defence responses, strongly reduced root colonization and abolished the benefits for the plants in a symbiosis between the endophytic fungus *Piriformospora indica* and *Arabidopsis thaliana* (Camehl et al., 2010). Thus the role of the ERF in colonization of plants by the beneficial bacteria could not be ignored. Repression of these ERF might be facilitating *Azospirillum* interaction with the *Arabidopsis*. The last two proteins (at3g11930 & at3g62550) in this pathway relate to class of universal stress protein (USP) family with unknown functions.

Auxins

Most of the genes falling in MAPMAN “auxin induced-regulated-responsive-activated”sub category are poorly characterized in the available on line resources. Concerning the gene regulation trend, six transcripts showed accumulated expression in treated samples while the rest of the four were repressed.. These proteins were shown to be responsive to the auxin stimulus. Still two of them (at4g31320 (SAUR-C) and at2g24400 (SAUR-D)), which are expressed in roots are predicted to be involved in calmodulin binding. Role of calcium in the plant *Azospirillum* relationship could not thus be over ruled. The only down regulated auxin responsive gene was a putative auxin-responsive GH3-type protein of unknown function.

AGI	Name	LFC
At5g53590	auxin-responsive family protein	1.30
At4g31320	auxin-responsive protein, putative / small auxin up RNA (SAUR_C)	1.79
At2g24400	auxin-responsive protein, putative / small auxin up RNA (SAUR_D)	1.33
At3g25290	auxin-responsive family protein	1.56
At5g10990	auxin-responsive family protein	1.46
At5g13370	putative auxin-responsive GH3-type protein (AtGH3.15)	-1.29
At2g33830	dormancy/auxin associated family protein	-3.60
At3g15450	hypothetical protein	-3.44
At4g27450	hypothetical protein	-1.97
At3g22850	hypothetical protein	1.29

Table 24 List of the auxin induced/regulated/responsive/ activated genes with log2 fold ratios.

We observed two other genes (at3g15450 & at2g33830) with severe reduction in expression after the bacterial treatment not only in the late time point (96hpi) but also 24 hours post inoculation. Unfortunately we were unable to trace functional characterization of these genes in different online databases.

Some other auxin related proteins like auxin biosynthetic and transport proteins, were also modified significantly upon *Azospirillum* inoculation. Since MAPMAN based classification allocated these genes in bins other than “Hormone metabolism”, they will be discussed accordingly.

3.2.3.4 Stress

The PGPR colonization of *Arabidopsis* affected a large number of both biotic and a-biotic stress associated genes. The results highlight 82 genes in the stress category with major changes in their expression pattern after being exposed to the bacterium *Azospirillum* Sp7.

Biotic stress

Significant and distinct changes were observed in 47 proteins related to biotic stress. There were 35 genes induced and 12 suppressed as a result of inoculation with Sp7. These proteins are further clustered in sub sub groups, noteworthy of which are discussed below.

Biotic stress-plant defensins

The only family of PR proteins affected by the bacterial treatment in our study was plant defensins (PDF). Plant defensins are small, basic peptides which owe their name from structurally related defensins found in other organisms, including humans (Thomma et al., 2002). Interestingly and unlike in the plants interaction with pathogenic bacteria, the marker protein PR1 and many such others remained unaffected upon Sp7 inoculation except for the defensins. The plant defensins are primarily known for their antifungal activity; none of the defensins has shown a down regulation trend in our results.

Our investigations show an induction trend for seven of the defensins and defensin-like proteins. Besides, two other defensins (PDF 1.2b & PDF 2.3) manifested higher expression levels but were less than the cutoff of LFC 1.

AGI	Name	LFC
At5g44420	Plant defensin 1.2a (PDF 1.2a).	2.28
At2g26020	Plant defensin 1.2b (PDF 1.2b).	0.99
At5g44430	Plant defensin 1.2c (PDF 1.2c).	1.29
At2g26010	Plant defensin 1.3(PDF 1.3).	1.48
At2g02120	Plant defensin 2.1(PDF 2.1).	1.39
At5g63660	Plant defensin 2.5(PDF 2.5).	2.21
At2g02130	Plant defensin 2.3(PDF 2.3).	0.95
At5g42235	Putative defensin like protein	1.63
At4g13235	Putative defensin like protein- EDA21-embryo sac development arrest 21	1.14

Table 25 Differentially regulated defensins with log2 fold ratios

PDF 1.2a (at5g44420), encodes an ethylene and jasmonate responsive plant defensin whose transcript levels seem not to be responsive to salicylic acid treatment. Besides, reports are available showing the induction of AtPDF1.2a, b, c and AtPDF1.3 upon Zn^{2+} treatment, but not of AtPDF1.4 and AtPDF1.5 (Mirouze et al., 2006). The pattern of regulation observed in our case demonstrated higher expression levels of AtPDF1.2a, b, c and AtPDF1.3 after Sp7 treatment. This might throw some light on the way Zn enhanced availability is ensured to plants on PGPR inoculation. Up regulation of PDF1, 2a at the same time predict SA independent pathways used by *Azospirillum* to defend plant against microbes. Other defensins have not been investigated in detail.

Biotic stress - other proteins

The bulk of remaining genes were less studied and functions they carry out were poorly determined. In nutshell, different pathogenesis related (PR) proteins, NADPH oxidases, Mildew resistance locus proteins (MLO), chitinases, R genes and some individual genes were seen with modified expressions.

The PR proteins with enhanced expressions lacked functional details. Similarly PR proteins exhibiting reduced expressions upon challenge by Sp7 were not fully investigated. A comparatively better studied PR5 is shown to be responsive

to UV-B and also involved in systemic acquired resistance in *Arabidopsis*, a process attributed to pathogen plant interaction.

Interestingly, a few proteins were identified with nucleotide-binding (NB) sites and leucine-rich repeat (LRR) domains, characteristic feature of R-genes (Martin et al., 2003) . Two phloem proteins ATPP2-A6 & ATPP2-A8 specified as R genes were induced upon association with PGPR *Azospirillum*. They both are predicted to function in signal transduction, defense response and innate immune responses.

Production of Reactive Oxygen Species (ROS), among others, is linked to the recognition of PAMPS by the plant. We observed two Respiratory Burst Oxidase Homologs (RBOHA & RBOHC) in our data significantly up regulated after being treated with Sp7. Both need phosphorylation and Ca-binding for activity. RBOHC is required for normal root hair elongation. The root architectural changes brought about by *Azospirillum* inoculation might thus involve the enhanced activity of this gene.

AGI	Name	LFC
At4g01700	chitinase, putative	1.35
At2g43570	chitinase, putative	2.56
At2g43610	chitinase, putative	1.19
At1g56680	chitinase, putative	1.79
At4g19810	chitinase, putative	1.26
At4g19760	chitinase, putative	1.57
At2g19970	putative PR-1-like extracellular protein of unknown function	2.65
At4g33730	pathogenesis-related protein, putative	1.35
At4g36010	pathogenesis-related thaumatin family protein	1.21
At2g19990	PR-1-LIKE	2.19
At1g75040	PR5	-1.03
At1g75030	THAUMATIN-LIKE PROTEIN 3(ATLP-3)	-1.46
At1g73620	thaumatin-like protein, putative / pathogenesis-related protein, putative	1.81
At5g07390	ATRBOHA	1.24
At5g51060	ATRBOHC alias RHD2	1.26
At3g03480	putative BAHD-type acyltransferase	-1.18
At5g45080	ATPP2-A6-Phloem protein 2	1.09
At5g45070	ATPP2-A8-Phloem protein 2	2.09

Table 26 Differentially regulated biotic stress related proteins with log2 fold ratios

Abiotic stress

Micro array analysis of this data bin pointed towards 35 genes in the inoculated samples exhibiting distinct regulation trends. A group of proteins pertaining to "Heat" sub sub class were one of the most prominent differentially regulated transcripts.

A biotic stress-heat

Analysis of array data revealed a class of heat shock binding proteins whose expression intensities declined after inoculation with the PGPR Sp7. Eight out of nine genes with amended regulation pattern was found to be under expressed. At2g42750, a DNAJ heat shock N-terminal domain-containing protein was the only induced gene. Two among the repressed genes ATHSFA2 (at2g26150) and HSP70 (at3g12580) were relatively better characterized. The later is shown to be induced upon exposure to pathogen (Noel et al., 2007), H_2O_2, heat and Cd^{2+}. The ATHSFA2, which is a transcription factor, is also shown to be responsive to chitin, H_2O_2 and heat.

AGI	Name	LFC
At1g80920	JB-heat shock protein binding-protein folding-chloroplast	-1.87
At2g17880	heat shock protein binding-protein folding-chloroplast	-1.39
At4g13830	J20 (DNAJ-LIKE 20)- heat shock protein binding-protein folding	-1.11
At1g56300	DNAJ heat shock N-terminal domain-containing protein-HSP binding- protein folding	-1.12
At3g12580	HSP70-response to pathogen, H_2O_2,Cd, heat	-1.03
At4g36040	DNAJ heat shock N-terminal domain-containing protein-HSP binding-	-2.37
At1g72416	HSP	-1.39
At2g26150	ATHSFA2	-0.84

Table 27 Differentially regulated a biotic stress related proteins with log2 fold ratios

3.2.3.5 RNA

There were eighty eight (88) proteins distinctly regulated upon Sp7 inoculation in the RNA class and bulk of them fell in the sub class RNA regulation of transcription.

RNA regulation of transcription

Expression patterns of considerable numbers of genes (82) were modified in our studies. These included an equal number of up & down regulated genes (41 each). Most of the transcripts were classified as transcription factors (TFs) which among themselves were divided in different families of TFs. Most distinctly emerging families in our array analysis are discussed below.

Basic helix loop helix (BHLH)

The basic/helix-loop-helix (bHLH) proteins are a super family of transcription factors that are involved in many plant cell and tissue developmental processes (Heim et al., 2003). As a result of inoculation with *Azospirillum*, basic Helix-Loop-Helix family (bHLH) of transcription factors emerged as the most prominent class of proteins where a considerable number of differentially expressed TFs was found related to root hair development. Upon mining of the available web based data for these TFs, it was revealed that that AtRHD6 (at1g66470) and AtRSL1 (at5G37800) are specifically required for the development of root hairs and act downstream of the genes that regulate epidermal pattern formation (Menand et al., 2007). Another study indicated two other BHLH TFs (at4g30980 and at5g58010) functioning in regulating root hair development in *Arabidopsis* (Karas et al., 2009). Another BHLH family protein (at1g27740) is predicted to be involved in root hair initiation and responsive to auxin stimiulus. Higher activity of these proteins in the inoculated samples might be responsible for changes in the architecture of root hairs in Col-0/Sp7 association.

BHLH038, *BHLH039*, *BHLH100* and *BHLH101* (comprising a subgroup of *BHLH* Ib genes) have been shown to be induced by iron deficiency and specific metal treatments like nickel, high zinc or high copper and was repressed upon application of high iron, low copper and low zinc (Wang et al., 2007). Besides AtbHLH38 and AtbHLH39 are involved in the iron deficiency induced synthesis and excretion of ribo-flavin or vitamin B2 (Vorwieger et al., 2007). Interestingly the denoted four members of the BHLH TF subgroup are among the genes with highest LFC in their expression when challenged with *A. brasilense*. The

riboflavin availability might be one of the factors for ensuring increased colonization of the bacteria to plant roots.

AGI	Name	LFC
At5g37800	AtRSL1	1.17
At1g05805	basic helix-loop-helix (bHLH) family protein	-1.17
At4g30980	basic helix-loop-helix (bHLH) family protein	2.41
At2g42280	basic helix-loop-helix (bHLH) family protein	-1.02
At1g27740	basic helix-loop-helix (bHLH) family protein	1.65
At3g56980	BHLH039	4.51
At5g04150	BHLH101	3.6
At3g56970	BHLH038	5.27
At1g66470	RHD6- basic helix-loop-helix (bHLH) family protein	1.44
At5g43175	basic helix-loop-helix (bHLH) family protein	1.30
At3g47640	basic helix-loop-helix (bHLH) family protein	1.69
At2g41240	BHLH100	5.9
At5g65320	basic helix-loop-helix (bHLH) family protein	1.02
AT5G58010	basic helix-loop-helix (bHLH) family protein	0.98

Table 28 Log2 fold ratios for BHLH TF family proteins

MYB domain containing TFs

We observed ten MYB domain containing and three MYB related genes with altered expression levels during inoculation with *Azospirillum*. Seven of them emerged with decreased transcripts while six showed increased expression. Most of the genes (induced) belonged to the R2R3 factor sub gene family which is the best studied among the MYB TF class. Members of this family have been shown to take part in processes like defense, symbiosis etc. One member of R2R3 gene family MYB72 (at1g56160) has an established role in induced systemic resistance (Segarra et al., 2009; Van der Ent et al., 2008). The same was found to be induced significantly in the treated plants, pointing towards induction of resistance in plants against pathogenic bacteria upon prior inoculation with *A. brasilense*. MYB90 (at1g66390), another highly expressed gene in our data set is postulated to be responsible for anthocyanin pigment production in plants. Anthocyanins are pigmented flavonoids that are responsible not only for coloration in plants but also had roles as antibiotics against microbial pathogens, insect repellents and signaling molecules in plant-microbe interactions (Cohen et al., 2002; Hahlbrock and Scheel, 1989).

Interestingly, a MYB2-like gene which acts as a transcriptional repressor and negatively regulates the biosynthesis of anthocyanins was repressed in treated plants. Taken together, the behavior of MYB90 and MYBL2 after exposure to Sp7 suggests enhanced production of anthocyanins pointing to their presumed role as signals in plant Sp7 interaction and in strengthening the defence of plant against deleterious microbes.

In addition to MYBL2, diminishing expression was recorded for six other MYB domains containing TFs. Few of these down regulated TFs such as AtMYB93 (at1g34670), MYB59 (at5g59780), MYB11 (at3g46130) and MYB3 (at1g22640) are postulated to show response to different hormones like SA (Yanhui et al., 2006). MYB3 besides its aforementioned presumed function also seems to carry out repression of phenyl propanoid biosynthesis. Its own decreased expression therefore is suggestive of induced phenyl propanoid (PP) biosynthesis. This theme is endorsed by increased activity of the genes directly involved in biosynthesis of PPs in our data (discussed later).

AGI	Name	LFC
At3g23250	MYB15- R2R3 factor gene family	1.68
At1g34670	AtMYB93- R2R3 factor gene family	-1.42
At5g14340	AtMYB40- R2R3 factor gene family-	1.87
At5g59780	MYB59.	-1.47
At3g12820	AtMYB10- R2R3	1.64
At1g57560	AtMYB50- R2R3	1.35
At3g46130	MYB111	-1.39
At1g56160	MYB72- R2R3	2.15
At1g22640	MYB3	-1.65
At1g66390	MYB90	1.29
At1g19510	*ARABIDOPSIS* RAD-LIKE 5	-1.17
At1g71030	MYBL2	-2.18
At1g18330	EPR1 (EARLY-PHYTOCHROME-RESPONSIVE1	-1.80

Table 29 Log2 fold ratios for MYB TF family proteins

AP2/ EREBP family of TFs

The genome wide scans of the *Arabidopsis thaliana* transcripts after treatment with *A .brasilense* resulted in change of the expression levels of different transcription factors families. The AP2/EREBP, APETALA2/Ethylene-responsive element binding protein family chiefly manifested reduced expression levels in

the inoculated plants. We came across a total of nine genes with changed expression out of which only one was with higher expression intensities. The rest generally depicted a down regulation trend when compared with the non-treated plants for the same set of genes. Majority of these resulting TFs in our data are not well characterized, still few got defined roles. For example at1g13260 (RAV1) is a putative DNA binding protein with two distinct types of DNA binding domains, AP2 and VP1/B3 is postulated to be involved in timing of flower flowering (Jofuku et al., 1994). Besides, it also acts as a negative regulator of *Arabidopsis* lateral root and rosette leaf development (Hu et al., 2004). Repression of this gene gives us an idea towards the mechanism responsible for known higher lateral root emergence after inoculation with the bacteria under investigation. Other genes with known function were at3g61630 (CYTOKININ RESPONSE FACTOR 6 or CRF6) and at5g05410 (DREB2A) a transcription activator involved in drought stress tolerance. at5g13910 seemed to be the only induced AP2 domain containing transcription factor which is functionally characterized and act as a positive regulator of GA-induced germination.

AGI	Name	LFC
At1g46768	RAP2.1-member of the DREB subfamily A-5 of ERF/AP2 TF family	-1.31
At5g19790	RAP2.11- member of the ERF subfamily B-6 of ERF/AP2 TF family	1.73
At1g79700	ovule development protein-TF activity.	-1.61
At1g13260	RAV1	-1.79
At3g61630	CRF6	-1.83
At5g13330	subfamily B-4 of ERF/AP2 transcription factor family.	-1.16
At5g05410	(DREB2A)	-1.5
At5g13910	Leafy petiole (LEP)-B-1 of ERF/AP2	1.78
At1g75490	DREB subfamily A-2 of ERF/AP2	-1.86

Table 30 Log2 fold ratios for AP2/ EREBP family of TFs

3.2.3.6 Miscellaneous

It is routinely observed that maximum number of genes with differential expression after microarray analysis fall into “Not assigned” & “Miscellaneous”

categories. However in the further detailed probe, they are occasionally duly considered. We, nevertheless, tried to treat these sections at par with the rest.

There were a total of 140 genes with altered expressions that were spread over different sub classes. Some of which are discussed here.

Glutathion S transferases (GSTs)

Glutathione S-transferases (GSTs) encoded by a large gene family, represent an important group of glutathione-(GSH) dependent detoxifying enzymes in plants. Our results depicts that the GSTs in the MAPMAN bin miscellaneous, emerged as a prominent class of proteins being affected by Sp7 treatment. Like defensins, all differentially expressed GSTs manifested an upward trend of regulation as compared to control plants. Pertinent to mention here that some detoxification enzymes which are encoded by a set of GST genes are activated by methionine-derived isothiocyanates via redox signaling, which is one reason for the cancer-preventive properties of Brassica-based diets (Talalay and Fahey, 2001). Isothiocyanates on the other hand, have been shown to act as major volatiles after challenging *Arabidopsis* with oxidative stress (Vercammen et al., 2001).

Almost al GSTs with altered transcripts upon *Azospirillum* inoculation either relate to the phi or tau class of *Arabidopsis* GST. These classes shared an equal number of differentially regulated genes (5 each). Most of them are suggested to function in toxin catabolic processes and have glutathione transferase activity. Among the relatively better investigated are AtGSTF7 (at1g02920) and ATGSTF10 (at2g30870), putative class phi GSTs having glutathione transferase and copper ion binding activity. They are supposedly engaged in responses to cadmium ion and toxin catabolic processes. AtGSTF7 also plays a predicted role in bacteria and fungi defense responses as well as salt stress (Dixon et al., 2002), besides, being the only GST with induced transcripts in all three time courses of Sp7 inoculation.

AGI	Name	LFC
At5g02780	In2-1 protein. putative	2.50
At1g02920	AtGSTF7 *Arabidopsis thaliana* Glutathione S Transferase PHI 7	1.83
At5g17220	AtGSTF12 *Arabidopsis thaliana* Glutathione S Transferase PHI 12	1.95
At3g62760	ATGSTF13 *Arabidopsis thaliana* Glutathione S Transferase PHI 13	2.11
At2g30870	ATGSTF10 *Arabidopsis thaliana* Glutathione S Transferase PHI 10	1.22
At1g49860	AtGSTF14 *Arabidopsis thaliana* Glutathione S Transferase PHI 14	2.58
At1g78370	AtGSTU20 *Arabidopsis thaliana* Glutathione S Transferase tau20	1.43
At1g27140	AtGSTU14 *Arabidopsis thaliana* Glutathione S Transferase tau14	1.69
At1g74590	AtGSTU10 *Arabidopsis thaliana* Glutathione S Transferase tau10	1.82
At2g29440	AtGSTU6 *Arabidopsis thaliana* Glutathione S Transferase tau 6	1.29
At1g14550	anionic peroxidase. putative	1.61
At1g14540	anionic peroxidase. putative	1.81

Table 31 Log2 fold ratios for GSTs

Oxidases and nitrilases

While going through the detailed annotations of differentially regulated genes of the subject class in a web based search we came across some interesting findings described below. To mention first, at least three enzymes with distinctly induced expression were identified implicated in aliphatic glucosinolates biosynthesis. The enzymes designated as Flavin Monooxygenase Glucosinolate S-Oxygenase (FMO GS-OX1 to FMO GS-OX3) re catalyze of the S-oxygenation of methylthioalkyl to methylsulfinylalkyl glucosinolates. The higher expression of these enzymes upon challenge with *A. brasilense* adds a new dimension to the already known diverse biological functions.

In the treated samples two nitrilases NIT1 and NIT3 manifested an up-regulation trend. However the p value for NIT1 was > 0.05 (0.09). Nitrilases have been shown to catalyse the hydrolysis of nitriles producing carboxylic acid and ammonia. Nitriles from the breakdown products of glucosinolates have been shown to be the preferred substrates of nitrilases (Vorwerk et al., 2001).

These nitriles are further converted to indole acetic acid (IAA). We can thus presume that the enhanced auxin biosynthesis in the inoculated *Arabidopsis* plants adopts the above mentioned route which might further co-relate to the increased root growth. This view is supported by the down-regulation of AMIDASE 1 enzyme which has been implicated in IAA synthesis from indole-3-acetamide (IAM) (Pollmann et al., 2003). Remaining proteins in this group were not functionally characterized.

AGI	Name	LFC
At1g62540	Flavin Monooxygenase Glucosinolate S-Oxygenase2 (FMO GS-OX2)	1.31
At1g62560	FMO GS-OX3	1.26
At1g65860	Flavin Monooxygenase Glucosinolate S-Oxygenase1 (FMO GS-OX1)	1.63
At3g44320	NITRILASE 3 (NIT3)	1.30
At1g08980	AMIDASE 1 (AMI1)	-1.30
At2g17720	Oxidoreductase. 2OG-Fe(II) oxygenase family protein	1.10
At3g43670	Copper amine oxidase. putative	2.01
At5g37980	NADP-dependent oxidoreductase. putative	1.79
At4g12290	Copper amine oxidase. putative	-1.16
At4g15760	MO1 (MONOOXYGENASE 1)	-1.58
At1g31690	Copper amine oxidase. putative	1.53
At1g26400	FAD-binding domain-containing protein	2.81

Table 32 Log2 fold ratios for oxidases and nitrilases

Despite the fact that some gene families like cytochrome P450 monooxygenase were quite prominant in the “Miscellaneous” bin they unfortunately lacked functional annotations. Besides cytochromes discussed elsewhere, only one Cyp450 here was found better investigated. This enzyme CYP81D1 has been found to promote volatile synthesis when induced. This gene was, however, down regulated in our experiments.

Others

Glycosyltransferases (GTs) are carbohydrate-active enzymes that add sugars to diverse substrates making them more stable. Sp7 treated array data analysis revealed a number of such proteins with modified expression. At4g14090 and at1g06000 are among the better functionally annotated genes and are involved in flavonoid biosynthesis (Tohge et al., 2005; Yonekura-Sakakibara et al.,

2007). As seen for the other flavonoid synthesis related proteins, these two also showed increased transcripts after being challenged with the PGPR Sp7. This further strengthens the notion of the predicted involvement of flavonoids in *Arabidopsis* association with *Azospirillum*.

BGLU21 & BGLU22 are beta glucosidases hydrolyzing O-glycosyl compounds. They are reported to hydrolyze scopolin (Ahn et al., 2010), a glucosylated derivative of scopoletin, a well known coumarin with antimicrobial properties. The enzymes in question exhibited up-regulation as a result of Sp7 colonization. Some galactosidases were found to be involved in cell wall modification dynamics of *Arabidopsis thaliana*. For instance the significantly repressed gene BGAL4 in our study is predicted to play a role in cell expansion during pollen germination (Hruba et al., 2005). Another repressed beta galactosidase in our study, besides being responsive to oxidative stress, is supposed to be implicated in cell wall modification.

AGI	**Name**	**LFC**
At3g28740	cytochrome P450 monooxygenase (CYP81D1)	-1.29
At4g14090	anthocyanin 5-O-glucosyltransferase family protein	1.74
At1g66270	putative beta-glucosidase BGLU21	1.73
At1g66280	putative beta-glucosidase BGLU22	1.47
At5g56870	beta-galactosidase 4 (BGAL4)	-2.22
At3g13750	Beta galactosidase 1 (BGAL1);	-1.96
At4g31970	CYP82C2 cytochrome P450 monooxygenase	2.51
At4g31950	CYP82C3 cytochrome P450 monooxygenase	2.11
At3g03470	CYP89A9 cytochrome P450 monooxygenase	-2.02
At4g20240	CYP71A27 cytochrome P450 monooxygenase	1.73
At3g26220	CYP71B3 cytochrome P450 monooxygenase	-1.69
At5g05900	UDP-glucoronosyl/UDP-glucosyl transferase family protein	1.40
At4g21760	Beta-glucosidase 47 (BGLU47)	1.98

Table 33 Log2 fold ratios for various genes in Misc. MAPMAN bin

3.2.3.7 Signalling

The array analysis demonstrated “Receptor kinases” and “Signalling calcium” as two leading sub bins under signaling pathway emerged upon *Azospirillum* inoculation.

Receptor kinases

Receptor like kinases (RLKs) gene family is the largest in *A. thaliana* comprising more than 600 members. Members of this family are involved in a wide range of signaling networks and developmental processes. The majority of the RLKs appearing with altered transcripts in our studies turned out to exhibit undefined roles. A protein kinase family protein (at4g23270) was among the few exceptions and was implicated in regulation of cell death after being induced by SA or pathogens (Chen et al., 2004). Noteworthy the same kinase was down regulated in our experiments showing a SA independent signature and avoiding the ultimate cell death, characteristic feature of pathogenic infection. Similarly a putative transmembrane protein kinase (at3g54590) which is up-regulated in this study is suggested to function in plant-type cell wall organization.

Among the up regulated genes, at5g37450 and at2g36570 exhibited the highest transcript accumulation. While the later is putatively connected in regulation of certain cellular processes that lead to cell elongation and expansion, no function could be traced for the earlier.

AGI	Name	LFC
At5g48940	leucine-rich repeat transmembrane protein kinase, putative	1.27
At4g28650	leucine-rich repeat transmembrane protein kinase, putative	1.44
At4g23270	protein kinase family protein	-1.03
At1g70530	protein kinase family protein	-1.24
At1g70460	Proline rich extension like RK	1.51
At3g54590	HYDROXYPROLINE-RICH GLYCOPROTEIN (ATHRGP1);	1.24
At2g48010	RKF3 (RECEPTOR-LIKE KINASE IN FLOWERS 3)	1.68
At2g36570	leucine-rich repeat transmembrane protein kinase, putative.	1.73
At5g49770	leucine-rich repeat transmembrane protein kinase, putative	1.33
At5g37450	leucine-rich repeat transmembrane protein kinase, putative	2.15
At1g63600	protein kinase family protein	1.20
At3g46370	leucine-rich repeat transmembrane protein kinase, putative	-2.17
At1g05700	leucine-rich repeat transmembrane protein kinase, putative	1.46

Table 34 Log2 fold ratios for distinctly regulated receptor kinases

3.2.3.8 Not assigned

Unlike a number of previous array studies, we by using the available online *Arabidopsis* resources, thoroughly screened the proteins in the not assigned MAPMAN bin.

Occurrence of sulfate groups is hallmark of various plant secondary metabolites like sulfoflavonoids and the glucosinolates. Two sulphotransferase showed induction upon colonization by Sp7. These proteins (sulphotransferase 17 & 18) are involved in catalyzing the final step in the biosynthesis of the methionine derived glucosinolates (Piotrowski et al., 2004). Furthermore they showed unchanged or very little change in expression when challenged with MeJA. Interestingly another sulphotransferase (at1g74100) exhibited significantly higher activity to MeJA. This protein remained, however, unaffected to Sp7 stimulus. It should be noted that the preferred substrate for the same protein is tryptophan, so it is part of the tryptophan derived GS core structure synthesis.

Two genes with higher expressions in the treated samples were related to trichome morphogenesis. at1g74030 which is a putative enolase, exhibited distorted trichomes and reduced numbers of root hairs in its T-DNA insertion line eno1 (Prabhakar et al., 2009). Mutants of the other induced gene at1g56580 gave rise to trichomes of smaller size with branches of variable length and number (Marks et al., 2009).

We traced at least eight genes with differential regulation pattern functioning in *Arabidopsis* defense. Colonization with *Azospirillum* severely reduced the transcript levels of two of these genes (at2g40000 & at2g15890). The earlier, in addition to be responsive to oxidative stress, also plays a role in defense responses to bacteria where it was negatively regulated by signaling through jasmonic acid and ethylene (Murray et al., 2007), highlighting thus the role of these hormones in plant *Azospirillum* associations. The other gene is reported to participate in defense responses to fungi in compatible interactions. A malate dehydrogenase (at3g15020) also manifested enhanced regulation in the Sp7 challenged plants. This protein is known to be involved in the defense response to bacteria. Three of the four remaining proteins are predicted to function in HR.

At5g50200 encodes a NAR2 like protein (AtNRT3.1) and is essential for high-affinity nitrate transport in *Arabidopsis* as it interacts with the nitrate transporter NTR2; 1 (Okamoto et al., 2006). Induction of both these transport-related genes after Sp7 inoculation might provide new insights into the molecular mechanism responsible for higher plant growth through availability of N to the plants.

We observed induction of two putative aluminum responsive malate transporters ATALMT3 & ATALMT5 in our array data. Although not characterized so far, a role of other member(s) of these kinds of transporters is well established in plant PGPR interaction (Rudrappa et al., 2008).

Seven proline-rich extensin-like family proteins were seen up regulated in the results. These proteins are structural constituents of the cell wall and are putatively implicated in cell wall organization. These proteins thus add to the already discussed genes of the same category.

AGI	Name	LFC
At1g53130	Grim reaper1 (GR1)	2.58
At1g74030	ENO1	1.55
At1g56580	hypothetical protein	1.16
At2g24980	proline-rich extensin-like family protein	1.47
At1g23720	proline-rich extensin-like family protein-	1.95
At3g28550	proline-rich extensin-like family protein	1.07
At3g49300	proline-rich family protein	1.7
At3g54580	proline-rich extensin-like family protein	1.37
At5g06630	proline-rich extensin-like family protein	-2.6
At5g06640	proline-rich extensin-like family protein	-2.73
At2g40000	*ARABIDOPSIS* ORTHOLOG OF SUGAR BEET HS1 PRO-1 2	1.31
At2g15890	(maternal effect embryo arrest 14)	1.39
At2g23960	defense-related protein, putative	1.14
At5g50200	(WOUND-RESPONSIVE 3)	1.09
At3g15020	malate dehydrogenase	1.36
At1g18420	Aluminum responsine malat transporter (ATALMT3)	1.55
At1g68600	Aluminum responsine malat transporter(ATALMT5)	1.16

Table 35 Log2 fold ratios for distinctly regulated genes in not assigned pathway

3.2.4 Common Genetic responses in all three time points

By looking at the given below Venn diagram, 14 genes are shown common to all time points in our array analysis results. Similarly four and twenty genes are shared between 6hpi & 96hpi and 24hpi & 96hpi respectively. However, none of the differentially regulated gene was jointly held between 6hpi & 24hpi.

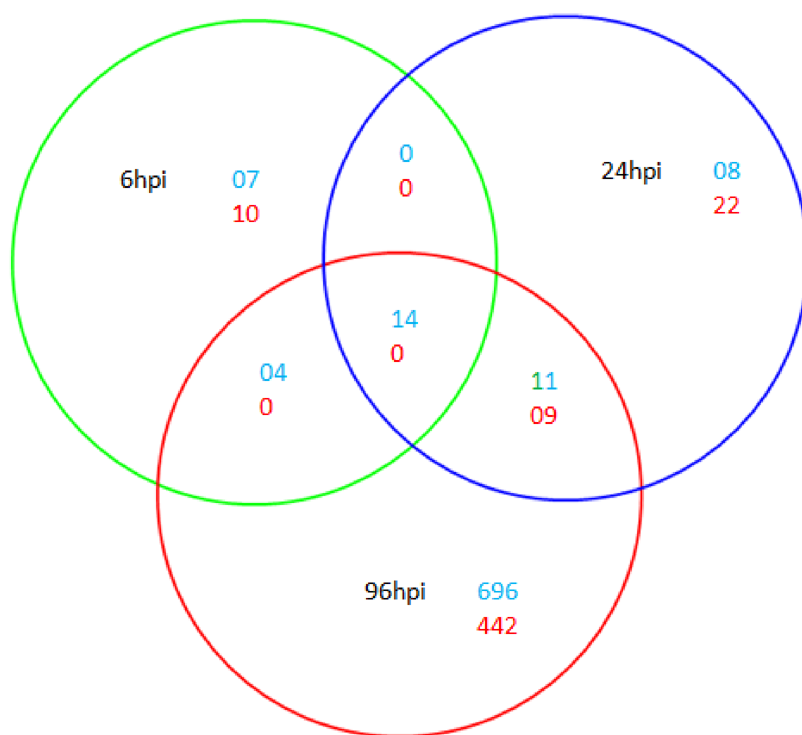


Figure 18 Venn diagram showing number of regulated genes in three time points after *Azospirillum* inoculation.

The genes that share common differential regulation in all time points are interestingly all up regulated. A putative PR protein having more than 40% identity to the known PR1, exhibits increase in its expression over time course ranging from 6 to 24 hpi. However, in 96hpi the induction ratio is almost the same as in 24hpi. Two other proteins (at1g26410 & at4g28850), manifested increase in their transcripts in a post inoculation time dependent manner. The later corresponds to xyloglucan endotransglucosylase-hydrolase (AtXTH26/AtXTR18) and is documented to play a role in the regulation of root hair morphology. This pattern of induction relates to the changes in root hair morphology resulting from *Azospirillum* inoculation. The oxidoreductase (at3g13610), on the other hand, was observed with pretty similar up regulation

trend in the early inoculation time zone but attained maximum transcript accumulation in 96hpi. Its role in plant defense via scopoletin formation seems to be elevated once *Azospirillum* is fully associated with the plant. Another defense related protein commonly up regulated in all time points was traced as a Glutathione *S*-transferases of phi class, which has a predicted activity in defense against pathogen and salt stress.

A germin-like protein (GLIP9), predicted to be implicated in manganese ion binding, nutrient reservoir activity and salt stress and a cell wall related putative proline-rich extension, which is structural constituent of cell wall with a putative role in cell wall organization showed relatively less activity in the 24hpi as compared to the early and late post inoculation regimes.

CYP81F2, a cytochrome P450, has been shown with roles in multiple plant defenses and indole glucosinolate metabolism. A series of publications establish its role in *Arabidopsis* innate immunity responses (Clay et al., 2009), in defense against the green peach aphid (Pfalz et al., 2009) and being part of the processes mediating broad spectrum antifungal defense (Bednarek et al., 2009). Induction of this gene upon *Azospirillum* treatment in all three time points studied yet explores another dimension of the role of this protein.

A Leucine-rich repeat putative protein kinase was identified with highest kinase activity in the 6hpi. We also came across another membrane protein of unknown function (at2g18690) and a photoassimilate responsive related protein (at3g54040) lacking any functional characterization. Their expression was significantly higher in the treated plants but similar at different time points post colonization.

An aspartyl protease family protein; having aspartic-type endopeptidase activity is predicted to be involved in proteolysis. However, conclusive experimental validation is still needed. In addition to this protease, three FAD domain containing proteins were also found being induced during the time course of this study. One of them (at1g32300) was one of the highest induced gene in the treated plants. Unfortunately we were unable to locate functional annotations of these genes.

AGI	Name	LFC		
		6hpi	24hpi	96hpi
At4g07820	PR protein, putative similar to PR1 (PATHOGENESIS-RELATED	1.86	2.71	2.65
At3g13610	Oxidoreductase	1.37	1.32	3.07
At1g02920	ATGSTF7 (GLUTATHIONE S-TRANSFERASE 11)	1.82	2.21	1.88
At3g54040	Photoassimilate-responsive protein-related	1.92	1.92	2.11
At4g14630	Germin-like protein (GLIP9)	2.09	1.59	3.67
At1g26250	Proline-rich extensin, putative	2.52	1.39	2.45
At4g28850	Xyloglucan:xyloglucosyl transferase, putative	1.35	2.29	3.19
At5g59680	Leucine-rich repeat protein kinase, putative	2.15	1.19	1.14
At2g18690	Unknown protein(transmembrane protein)	1.49	1.15	1.49
At1g26380	FAD-binding domain-containing protein	1.96	2.30	1.96
At1g32300	FAD-binding domain-containing protein	4.42	3.02	6.72
At1g26410	FAD-binding domain-containing protein	1.38	1.72	2.17
At5g57220	CYP81F2 (cytochrome P450, family 81, subfamily F, polypeptide 2)	1.63	2.31	1.63
At3g51350	Aspartyl protease family protein	1.81	1.38	1.95

Table 36 Log2 fold ratios for distinctly regulated genes common in all three time oints.

3.2.5 Comparative analysis

A brief comparison of the results obtained after different times of inoculation clearly indicates that the bulk of genes were differentially regulated in the late time period i.e. 96hpi. The number in this case exceeded one thousand with up-regulated genes surpassing the down-regulated ones. Only 99 proteins with differential regulation were seen in the other two classes.

In terms of percentage of total differentially regulated genes, more signaling and secondary metabolism related proteins were found in the early time point (6hpi). Similarly for the 24hpi, “hormone metabolism” and “cell wall” while in 96hpi period, MAPMAN classes namely “RNA”, “protein”, “transport” and “development” manifested the highest percentage of the total number of distinctly expressed genes. The percentage of genes falling in the stress class remained almost unchanged over the time course of the experiment (Figure 19). The obtained pattern very well fits the assumptions if considering the interaction of a PGPR with a plant where, for instance, signaling events predominantly are

expected earlier while activities such as development would show up at later time points. It may however be noted that a significant number of genes for different classes was only observed in the late post inoculation regime as evident from the table 37.

	6hpi	24hpi	96hpi
Differentially regulated genes	35	64	1174
Up-regulated genes	25	33	724
Down-regulated genes	10	31	450

Table 37 Number of differentially regulated genes in different time points

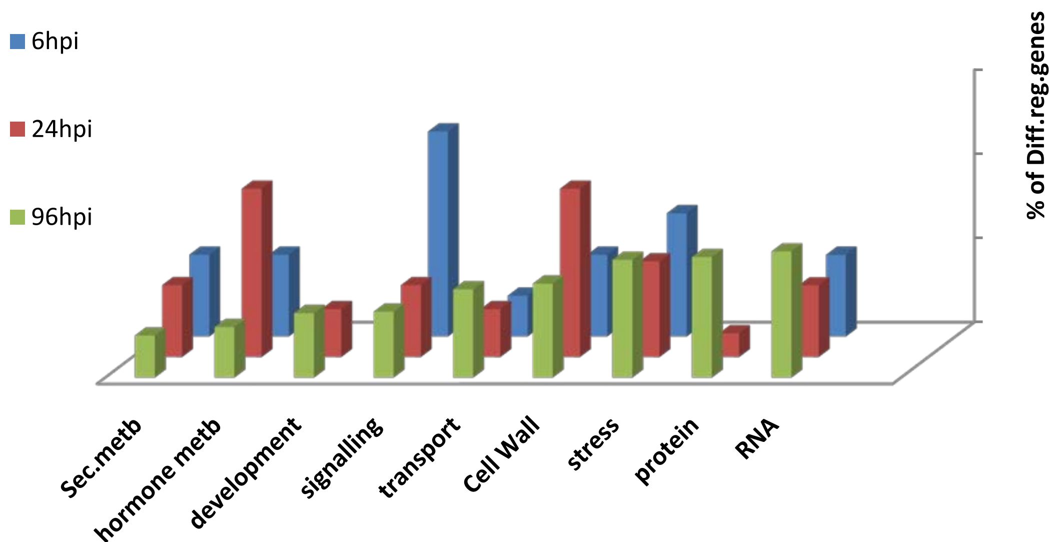


Figure 19 Comparison of the representative pathways among different time points after treatment with Sp7. The y axis represents percent of the genes differentially regulated in each time course

3.2.6 Validation of microarray results by qRT-PCR

The gene regulation profiles observed in the array results were validated by the qRT-PCR measurements. The expression values of the ftfteen representative genes, randomly selected from all time points, were found highly similar to the corresponding microarray results. The only exception was a putative remorin protein (only for 6hpi).The said gene was down regulated in the array data but this regulation trend could not be confirmed with the real time PCR results.The given table summarizes the comparison of array data to qRT results:

Gene	hpi	Array LFC	qRT-PCR LFC
AT1G08090	6	1,22*	1,08
High affinity nitrate transporter2.1	24	0.002	0,09
	96	2,79	3,69
At2g42060	6	1,6	2,19
CHP-rich zinc finger protein	24	< 1	< 1
	96	1,89	2,47
AT2G33830	6	< 1	< 1
dormancy/auxin associated family protein	24	-1,17	-1,16
protein of unknown function	96	-3,6	-3,12
At3g02040	6	< 1	< 1
AtSRG3 (senescence-related gene 3)	24	1,05	1,29
	96	< 1	< 1
AT3G13610	6	1,36	1,78
putative iron-(II)-dependent oxidoreductase	24	1,31	1,63
	96	3,76	4,47
At1g26380	6	1,96	2,14
putative reticuline dehydrogenase	24	2,63	2,84
	96	2,69	3,33
At2g18690	6	1,55	1,23
putative membrane protein of unknown function	24	1,14	1,1
	96	1,09	1,56

AT5G41300 receptor-like protein kinase-related protein	**6**	1,6	2,52
	24	< 1	< 1
	96	< 1	1,32
AT5G45070 disease resistance protein (TIR class), putative	6	< 1	< 1
	24	< 1	< 1
	96	2,09	3,6
At5g59680 putative receptor-like protein kinase	6	2,15	2,53
	24	1,18	1,41
	96	1,15	1,92
At4g25250 invertase/pectin methylest. inhibitor protein	6	< 1	< 1
	24	*-1,23*	-1,56
	96	*96*	1
AT4G14630 germin-like protein (GLP9)	6	*6*	2,08
	24	*24*	1,59
	96	*96*	3,76
At4g07820 pathogenesis-related protein, putative	6	1,85	2,08
	24	2,7	2,88
	96	2,64	3,21
At3g57540 remorin protein	6	-1,25	0,19
	24	< 1	< 1
	96	< 1	< 1
At3g54040 photoassimilate-responsive protein of unknown function	6	1,92	1,87
	24	1,1	0,9
	96	2,26	2,49

Table 38 Comparative analysis of the expression profiles of different genes based on the data obtained from microarray and qRT-PCR.The values depict the log (base2) fold change (LFC). * represents statistically non significant values

4 Discussion

The present study is an initiative in terms of exploring; the broader view of changes in *Arabidopsis* at the whole genome level upon association with *Azospirillum brasilense* and bacterial signals that are sensed by plants and may regulate plant gene expression. The project attains importance in light of the established beneficial effects of the bacterium on wide range of plants but largely unknown / unattended molecular processes underlying these positive effects. The results of the transcriptome profiling experiments are expected to lay a foundation for more specific and focused future investigations.

4.1 Early signalling events

In order to defend themselves from the invading pathogens, plants usually depend on a two branched innate immune response (Jones and Dangl, 2006). The first layer of such defenses utilizes transmembrane pattern recognition receptors (PRRs) to respond to the microbial signatures usually referred to as pathogen or microbe-associated molecular patterns (PAMPs or MAMPs) (Zipfel and Felix, 2005) while the other level uses nucleotide binding (NB) leucine rich repeat (LRR) domains containing proteins that are encoded by resistance (R) gene (Dangl and Jones, 2001). In case of PAMPs or MAMPs, the preferred recent trend favors use of MAMPs for these molecular patterns as they pertain to whole class of microbes irrespective of their pathogenic or mutualistic nature (Boller and Felix, 2009).

MAMPs trigger different plant responses upon perception. The extracellular alkalinization coupled with ion fluxes are among the earliest responses observed when plants are challenged with pathogenic microbes or the purified elicitors thereof (Atkinson et al., 1985; Felix et al., 1999). Although these findings were initially specified for plant pathogen interactions, similar results in subsequent work on Rhizobia-legume and PGPR plant association provoked many scientists to phrase that both antagonist and friendly microbes are initially perceived as invaders by the plants activating alike early responses (Soto et al.,

2009). While a plethora of such studies in context of plant pathogen exists, the PGPR plant partnership referring to early perception events is just in its infancy.

Despite the fact that *Azospirillum* imparts an evident growth promoting effect on a variety of plants, the early signaling events and the underlying plant responsive genes are largely unknown. We, therefore, attempted to provide insights into these mechanisms, focusing particularly on the similarities and differences with regard to plant pathogenic context.

Applying already existing models, we on one hand were able to detect apoplastic alkalinization responses of *Arabidopsis* cell cultures with live *Azospirillum* as well as with its bacterial preparations (lysates). The amplitude of response in terms of pH units was higher for the live bacteria (fig 20).

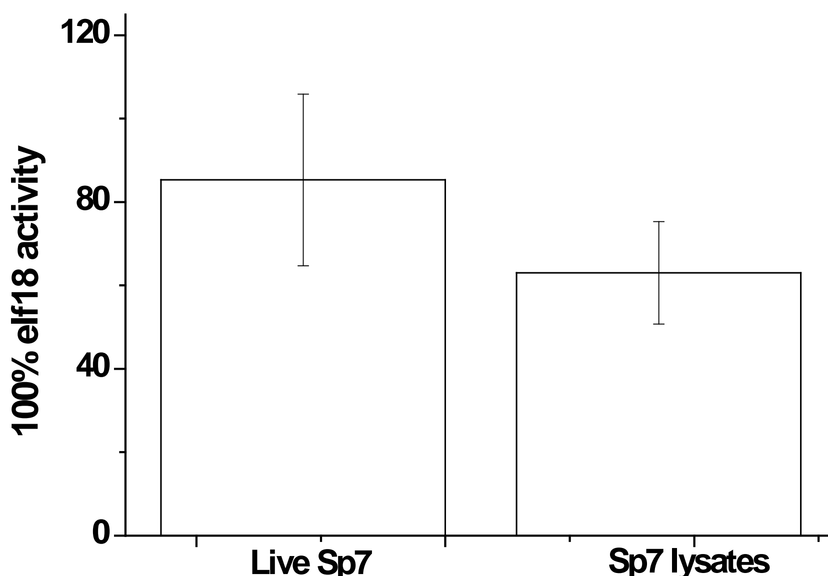


Figure 20 Comparative medium alkalinization activity of the live bacteria and bacterial lysates relative to 100% elf18 activity in *Arabidopsis* cell culture. Lysates were used at 5µg/ml of the suspension cell cultures while live bacteria grown up to 1.5 (OD600) were re suspended in 200µl of media and used at 3µl/ml. Data represent mean values of n = 4 ± SD

The reason for this difference might relate to the presence of multiple bacterial elicitors and their redundancy (especially in the bacterial cell wall) present in the live bacterium fraction but absent ifrom the lysates. Isolated flagella, however, were completely inactive even at high concentrations, excluding the possibility of some flagellar epitope as a factor being recognized by the plant.

The activity of lysates was determined to be of proteinaceous nature as protease treated lysates were unable to elicit medium alkalinization. Together with our observation of non responsive tomato cells to these lysates, the functional eliciting activity in *Azospirillum* lysates was supposed to be connected to the elongation factor proteins (EF-TU) fractions of *Azospirillum.* The premise was based on the fact that responsiveness to elf18/elf26 is shown to be limited to Brassicaceae species and many others including tomato are devoid of it (Kunze et al., 2004).

EF-TU which is among the most abundant and conserved bacterial proteins, owes its MAMP activity to its N terminus. Synthetic peptides matching the acetylated N terminus of EF-Tu, called elf18 and elf26, have the ability to generate MAMP responses in *Arabidopsis* (Kunze et al., 2004). In a later study, a functional receptor binding site in plants for EF-TU was recognized as elongation factor receptor (EFR) which upon binding to EF-TU triggers typical PAMP responses (Zipfel et al., 2006).

In this background we challenged both *efr* mutants and *Arabidopsis* ecotype Wassilewskija (WS-0) lacking the functional flg22 receptor (FLS2) with *Azospirillum* lysates and observed their response in terms of production of reactive oxygen species (ROS) in a luminal based assay.

The production of reactive oxygen species (ROS) is often linked to plant pathogen defense processes directing programmed cell death (Bolwell, 1999) and mediating the plant hypersensitive disease resistance response (Levine et al., 1994). Furthermore, its role in the systemic expression of defense-related genes is also evident (Orozco-Cardenas et al., 2001). However, ROS functioning in *Rhizobia*-legume interaction (Cardenas et al., 2008; Mathis et al., 2005), PGPR plant association (Rudrappa et al., 2007) and in plant root hair

growth (Foreman et al., 2003), are some other dimensions elucidating the diversified and imperative nature of these molecules.

Investigations with *Arabidopsis* (Col-0) leaf discs clearly demonstrated ROS production upon recognition of *Azospirillum* lysates. Furthermore, the non responsiveness of *efr* mutants strongly suggests the bacterial elongation factor as one of the elicitors being recognized by *Arabidopsis* plants. Besides, the *Arabidopsis* ecotype Wassilewskija (WS-0), despite of being insensitive to flagellin perception, was fully responsive when challenged by *Azospirillum* lysates. These results are in agreement with our pH assay outcomes, where the *Azospirillum* flagella could not elicit any response in *Arabidopsis* cell cultures but the bacterial lysates prompted the apoplastic pH changes. Although EF-TU has been suggested as one of the elicitor of *A.brasilense* recognized by the plant, the active domain of EF-TU still needs to be established.

The array data on the other hand predict a possible genetic basis for the production of ROS upon recognition of PGPR elicitor. Plasma membrane NADPH oxidases alias respiratory burst oxidase homologues (RBOH) are the most likely sources of ROS produced in the apoplast (Grant et al., 2000). Out of ten (10) RBOH proteins in *Arabidopsis*, *Azospirillum* infection probably affects RBOHC, RBOHA and RBOHG (0.96 LFC) as seen from their higher transcript levels in the treated seedlings. We could therefore suspect these proteins as possible sources of higher ROS in response to PGPR recognition. This is unlike pathogen plant interaction where ROS production is chiefly mediated by RBOHD and RBOHF (Pogany et al., 2009; Torres et al., 2002). Consistent with RBOHC role in *Arabidopsis* root hair development (Foreman et al., 2003), induction of this gene also suggests one of the underlying mechanism responsible for improved root architecture upon *Azospirillum* challenge. The inoculated plants also exhibited induction of CPK2 (0.86 LFC) and CPK4 (0.96 LFC) which may correlate to the Ca^{+2} dependent productions of ROS by RHD2 (RBOHC)(Takeda et al., 2008) ensuring the stimulated hair growth.

Despite the similarities in the processes related to early recognition of the bacterial elicitors, divergence seems to operate downstream of these early

events after elicitor recognition. For instance, decrease in disease virulence was observed by virtue of MAP kinase cascade and over expression of WRKY transcription factors downstream of flg22 perception (Asai et al., 2002) resulting in expression of the defense related genes like PR1, PR2 and PR5 (Gomez-Gomez et al., 1999). *Azospirillum* inoculation, unlike the pathogenic elicitors flg22 or elf18, does not seems to interfere with WRKY transcription factor and PR1 induction and interestingly imparts repression of WRKY26, WRKY75 and PR5 transcripts. Notably PR5 is considered as one of the marker genes in systemic acquired resistance (SAR).

The similarities and differences observed in early signaling cascades between the pathogenic and beneficial microbes probably indicates that the plant considers both as intruders in the first instance (Soto et al., 2009). However, the successful intimacy of the plant with mutualistic bacteria and non existence of mounted defenses might relate to the bacterial ability to interfere with the downstream recognition events through a yet unexplored mechanism.

Regarding postulating EF-TU as elicitor in a mutualistic scenario, it may be noted that elongation factor from *Sinorhizobium meliloti* has already been reported to induce apoplastic alkalinization in *Arabidopsis* cells (Kunze et al., 2004). Partial sequencing of the N terminus of the putative EF-TU gene from *Azospirillum brasilense* strain Sp7 was carried out. Alignments of the *Azospirillum* EF-TU N terminus sequence to those of various *Rhizobia*, PGPRs and pathogenic bacteria (http://www.ncbi.nlm.nih.gov/), revealed a high degree of similarity mainly among the mutualistic bacteria (fig21). A striking difference was observed at position 3 and 12 where most of the bacterial sequences differed to that of *A. brasilense*. The position 12 of *Azospirillum* EF-TU gene featured amino acid cysteine while the rest of the sequences exhibited valine at the similar position. Amino acid glycine at position 3 of *A. brasilense* was either substituted by alanine, glutamic acid or serine in different EF-TU sequences. The highest degree of similarity and difference of *Azospirillum* EF-TU N terminus sequence (first 35 amino acids) was observed with *Agrobacterium* and *P. syringae* respectively. These differences are also reflected in the potential of

these elicitors to trigger MAMP responses in *Arabidopsis* cell culture as the earlier proved to be a potent elicitor and the latter a very week one (Kunze et al., 2004). Whether or not these minor amino acid differences could account for significant differences in the plant genetic responses, is still to be resolved on the basis of mutagenized, recombinant proteins.

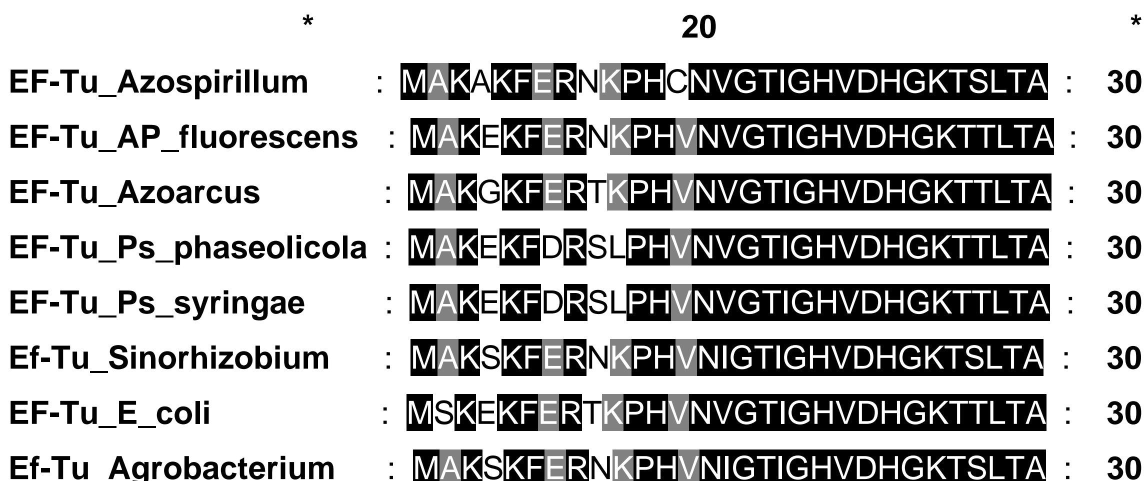


Figure 21 N terminal peptide sequences alignment of different bacterial EF-TU genes.

4.2 Genome wide studies - a comparative approach

Micro array analysis enabled us to broadly comprehend the *Arabidopsis* gene regulation trend after being inoculated with *Azospirillum*. In order to dissect changes which were more specific to *Azospirillum*, we carried out comparisons of our results with two already published different microarray data. One of these investigations highlights transcriptome profiling of *Arabidopsis* genome after treatment with flg22 (PAMP) and plant cell wall endogenous oligogalacturonides (OGs) (Denoux et al., 2008) a damage associated molecular pattern (DAMP). The second microarray study deals with the *Arabidopsis thaliana* genetic responses to *Agrobacterium tumefaciens* inoculation, which is a known causal agent of crown gall disease (Lee et al., 2009) . Rather than using the analysed files, raw data files of these two studies were put to identical analysis techniques as adopted in our data. In case of flg22 and OGs, ten days old

seedlings were treated for 1 & 3 hours. For *Agrobacterium* the results are for 3hpi & 6dpi (days post inoculation).

4.2.1 Genes with alike regulation trend

In an attempt to figure out gene groups exhibiting alike differential regulation patterns in all/most of the four array data under comparison, we observed very few genes in common. This in itself is indicative of the fact that different set of genes are regulated by *Azospirillum* as compared to other treatments.

A

Treatment	**Up regulated genes**	**Down regulated genes**
Azospirillum	740	484
C-58	150	119
Flg22	1159	766
OGs	553	45

B

Commonly regulated genes	All	Azospirillum/C-58	Azospirillum/flg22	Azospirillum/OGs
Total	18	47	103	2
Up regulated	12	17	56	1
Down regulated	6	29	47	1

Table 39 Tables showing summary of number of genes **a)** differentially regulated after different treatments and **b)** exhibiting alike regulation pattern between different treatments

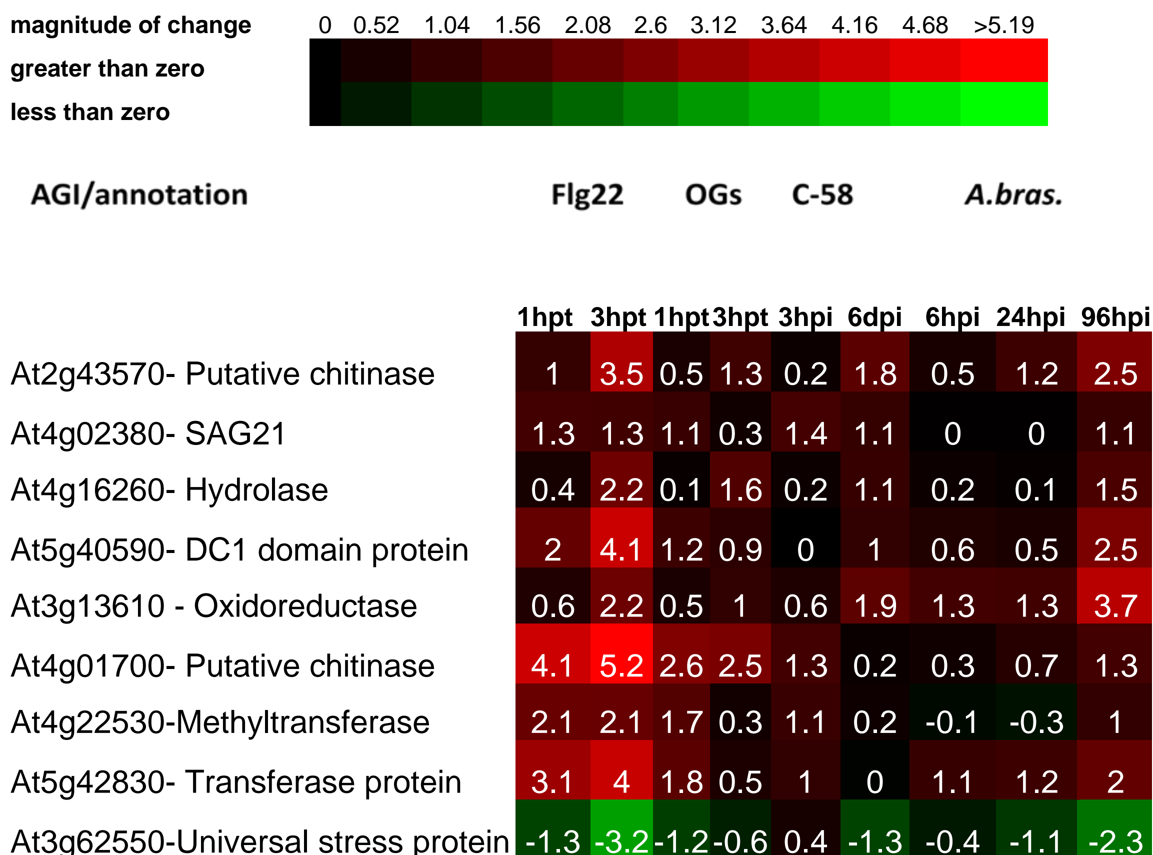
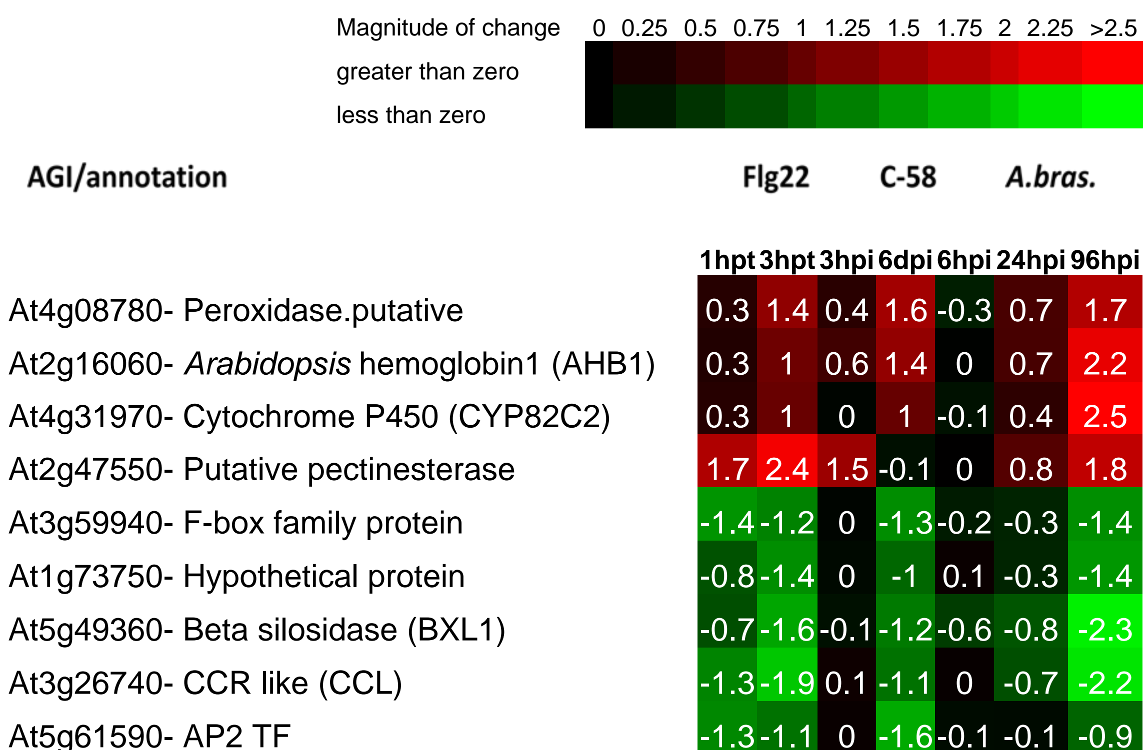


Figure 22 Some of the representative genes with similar differential regulation pattern in at least one time point of all array data studied. The heatmaps generation resource was http://bbc.botany.utoronto.ca/. The color scale represents log2 fold change

These genes could not be functionally clustered owing to divergent processes individual genes were involved in. Lack of functional annotation further hampered grouping these transcripts on functional grounds. The genes induced in all four array data in at least one time point of each of them, among others, included two putative chitinases (at2g43570 and at4g01700) predicted to be involved in cell wall macromolecule catabolic processes with localization in the cell wall. A somewhat better studied protein, Senescence associated protein 21(SAG21) is reported mediating protection against oxidative stress (Mowla et al., 2006). At4g16260, an hydrolase functions in salt stress (Jiang et al., 2007) while at3g13610, an oxidoreductase is involved in scopoletin biosynthesis in *A. thaliana* (Kai et al., 2008). The only gene showing repressed expression levels

in all arrays under consideration was at3g62550 relating to class of universal stress protein (USP) family with unknown function.

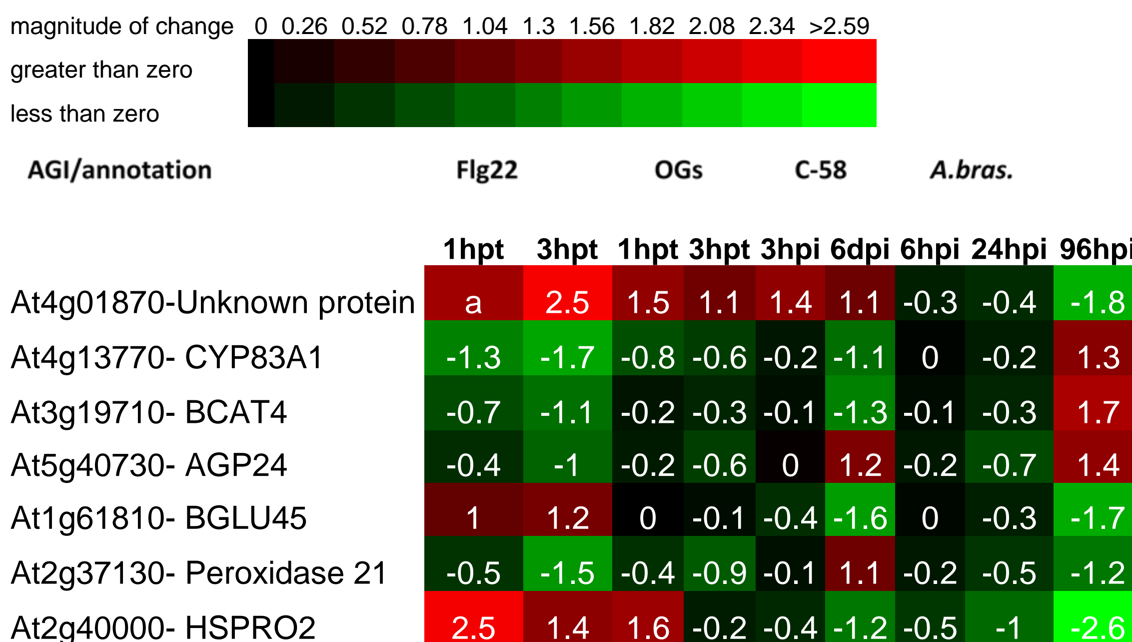
Some proteins were induced or repressed only by flg22, C-58 & *Azospirillum* treatments but not by OGs. The induced genes included a putative peroxidase (at4g08780), a pectinesterase family protein (at2g47550), an *Arabidopsis* HEMOGLOBIN 1 (at2g16060) protein and the cytochrome CYP82C2 (at4g31970). The latter two showed quite high induction in *Azospirillum* treated samples. Few genes, on the other hand, were commonly down regulated by these three treatments. One such gene BXL1 (BETA-XYLOSIDASE 1) is suggested to be expressed specifically in tissues undergoing secondary wall thickening and has a documented role in seed coat development (Arsovski et al., 2009).The rest of the proteins including at4g08780, a kelch repeat-containing F-box family protein, a CCL (CCR-LIKE) protein and an AP2 domain-containing transcription factor family protein (at5g61590) were mainly uncharacterized.



AGI/annotation	Flg22		C-58		A.bras.		
	1hpt	3hpt	3hpi	6dpi	6hpi	24hpi	96hpi
At4g08780- Peroxidase.putative	0.3	1.4	0.4	1.6	-0.3	0.7	1.7
At2g16060- *Arabidopsis* hemoglobin1 (AHB1)	0.3	1	0.6	1.4	0	0.7	2.2
At4g31970- Cytochrome P450 (CYP82C2)	0.3	1	0	1	-0.1	0.4	2.5
At2g47550- Putative pectinesterase	1.7	2.4	1.5	-0.1	0	0.8	1.8
At3g59940- F-box family protein	-1.4	-1.2	0	-1.3	-0.2	-0.3	-1.4
At1g73750- Hypothetical protein	-0.8	-1.4	0	-1	0.1	-0.3	-1.4
At5g49360- Beta silosidase (BXL1)	-0.7	-1.6	-0.1	-1.2	-0.6	-0.8	-2.3
At3g26740- CCR like (CCL)	-1.3	-1.9	0.1	-1.1	0	-0.7	-2.2
At5g61590- AP2 TF	-1.3	-1.1	0	-1.6	-0.1	-0.1	-0.9

Figure 23 Some of the representative genes with alike differential regulation pattern in at least one time point of different array data. Color scale shows log2 fold change

While considering mixed patterns of expression among the different array data, we traced two genes displaying a down slide trend in flg22 and C-58 treated plants but an up slide regulation pattern in *Azospirillum* inoculated plants (96hpi). Noteworthy, the two up-regulated genes (at4g13770 & at3g19710) were observed with established roles in glucosinolate biosynthesis (Schuster et al., 2006). On the other hand at4g01870 was the only entry found to be up regulated by all other treatments in both early and late time course and repressed in 96hpi of *Azospirillum* treatment. Search of data bases revealed the gene as a tolB related protein with no known functions. A beta glucosidase gene BGLU45 (at1g61810), suggested to function in liginin biosynthesis (Escamilla-Trevino et al., 2006) was induced by flg22 but repressed by both C-58 and *Azospirillum* inoculation. Another gene at2g40000, *Arabidopsis* ortholog of sugar Beet HS1 PRO-1 2 (HSPRO2) was very highly down regulated in the late time point of *Azospirillum* treated samples but up regulated with flg22. As discussed elsewhere this gene plays a role in defense response to bacteria in an SA dependent manner and is negatively regulated by jasmonic acid and ethylene signaling pathways (Murray et al., 2007)



AGI/annotation	Flg22 1hpt	Flg22 3hpt	OGs 1hpt	OGs 3hpt	C-58 3hpi	C-58 6dpi	A.bras. 6hpi	A.bras. 24hpi	A.bras. 96hpi
At4g01870-Unknown protein	a	2.5	1.5	1.1	1.4	1.1	-0.3	-0.4	-1.8
At4g13770- CYP83A1	-1.3	-1.7	-0.8	-0.6	-0.2	-1.1	0	-0.2	1.3
At3g19710- BCAT4	-0.7	-1.1	-0.2	-0.3	-0.1	-1.3	-0.1	-0.3	1.7
At5g40730- AGP24	-0.4	-1	-0.2	-0.6	0	1.2	-0.2	-0.7	1.4
At1g61810- BGLU45	1	1.2	0	-0.1	-0.4	-1.6	0	-0.3	-1.7
At2g37130- Peroxidase 21	-0.5	-1.5	-0.4	-0.9	-0.1	1.1	-0.2	-0.5	-1.2
At2g40000- HSPRO2	2.5	1.4	1.6	-0.2	-0.4	-1.2	-0.5	-1	-2.6

Figure 24 Some of the representative genes with mixed differential regulation pattern in different array data with a color code legend. The color scale represent log2 fold change

4.2.2 Unique gene regulation trend of *Azospirillum* treated samples

Further in depth scrutiny of all array data with respect to genes behaving differently in *Azospirillum* treated arrays in comparison to others resulted in some very interesting findings.

In the first instance, when down regulated genes in *Azospirillum* challenged plants were compared to the genes of the other three arrays; we came across 38 proteins which were up regulated in either of the other three array data. Although no specific functional grouping could be traced, still few transcripts are worth mentioning. For instance, the AZOSPIRILLUM-repressed At5g26340 encodes a protein with high affinity, hexose-specific/H+ symporter activity. It's over expression is shown to be linked to programmed cell death (PCD) (Norholm et al., 2006). PCD is usually related to pathogen plant interactions. Repression of its transcript levels by *Azospirillum* signifies PGPR-plant interaction differences to that of plant pathogen interactions. The WRKY75 (at5g13080) TF whose repression correlates with an increased number of lateral roots, root hairs and root length (Devaiah et al., 2007), exhibited up slide trend in both flg22 and OGs treated plants. In line with the observed root architecture phenotype connected to *Azospirillum* interaction with plants, WRKY75 exhibited repressed transcripts in PGPR colonized seedlings. The down regulation of this gene might thus be determinative for a role of WRKY75 in the mechanism of growth promotion by *Azospirillum*.

Strikingly, upon comparison of the up regulated genes in our results to the selected transcriptome profiles of *Arabidopsis* exposed to flg22, OGs and C-58, a specific fraction of proteins involved in the biosynthesis of aliphatic glucosinolates was observed with unique differential regulation pattern. This distinct regulation in *Azospirillum* treated plants is in contrast to decreased transcripts or no significant changes in other arrays under comparison. The said cluster of unique genes is shown to be involved in Met derived aliphatic glucosinolate biosynthesis pathway.

If we look at different proteins carrying out Met derived glucosinolate synthesis, it is evident that most of them were induced upon *Azospirillum* inoculation. For

instance, a branched chain aminotransferase, BCAT4 is known to catalyze the transamination reaction of Met in the cytosol (Schuster et al., 2006). The product 2-keto acid, 4-methylthio-2-oxobutanoate (MTOB) after being transported to chloroplasts by a bile acid transporter, BAT5 (Gigolashvili et al., 2009), MTOB engages in a condensation reaction with acetyl-CoA. Enzymes suggested to be responsible for catalysis of this event are methylthiomalate synthases MAM1 to MAM3 (Kroymann et al., 2001; Textor et al., 2004; Textor et al., 2007).Subsequent isomerization, de carboxylation, chain elongation and transamination steps take place in the plastid from where the chain elongated 2 keto acid is transported back to cytoplasm. The genes involved in all these processes are suggested to be target of the transcription factor MYB28. Interestingly an up regulation trend was observed for all genes except for BCAT3 implying that the second chain elongation step might take place in the cytoplasm.

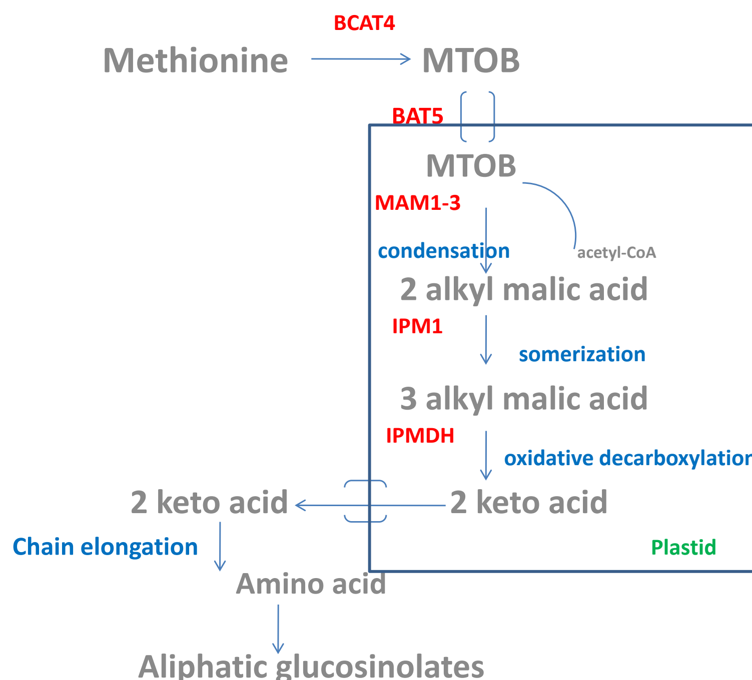
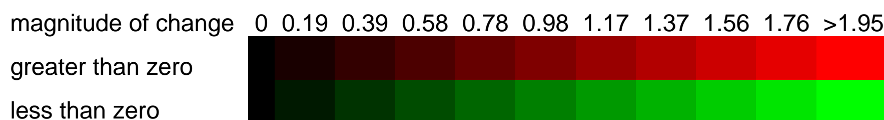


Figure 25 Simplified Met. derived aliphatic glucosinolates biosynthesis. The text in red indicates the genes involved in these particular steps

Similarly, during side chain modification, the resulting core methylthioalkyl glucosinolates from methionine chain elongation can further be modified. Certain Flavin-monooxygenases (FMO GSL-OX1-5) mediate the conversion of methylthioalkyl glucosinolates to methylsulfinylalkyl glucosinolates. Three of the five FMOs with significant higher regulation indicate the possible modification of the core glucosinolates structure to the succeeding products in *Azospirillum* treated samples. It is pertinent to mention here that the cancer preventive features of aliphatic glucosinolates are suggested to be linked to oxygenated glucosinolates (Juge et al., 2007), a scenario which imparts tremendous importance to FMOs responsible for secondary modification of glucosinolates via oxygenation.

Methylsulfinylalkyl glucosinolates could be converted to either alkenyl glucosinolates or hydroxyalkyl glucosinolates which are respectively controlled by AOP2 & AOP3 loci. While we could detect significant altered transcripts only for AOP2, seemingly methylsulfinylalkyl glucosinolates are chiefly converted to alkenyl glucosinolates in our case.

Specific antimicrobial activity of the glucosinolates is often linked to their hydrolyzed products like various nitriles and isothiocyanates. The functional alleles driving the process are specified at the ESP locus for nitriles and at ESM1 for isothiocyanates. Over expression of ESM1 represses nitrile formation and favors isothiocyanates production (Zhang et al., 2006). Viewing higher regulation of ESM1, we could expect major activity of isothiocyanates in *Azospirillum* challenged plants. The role of isothiocyanates, specifically 4-methylsulphinylbutyl isothiocyanates (ITC), is well known as an anti microbial agent in context of different fungal and bacterial pathogens (Tierens et al., 2001). The line of evidence suggests the predictive potential of use of *Azospirillum* to protect plants against a range of deleterious pathogens.



AGI/annotation	Flg22		OGs		C-58		*A.bras.*		
	1hpt	3hpt	1hpt	3hpt	3hpi	6dpi	6hpi	24hpi	96hpi
AT3G19710- BCAT4	-0.7	-1.1	-0.2	-0.3	-0.1	-1.3	-0.1	-0.3	1.7
AT5G23010 –MAM1	-0.9	-1.1	-0.4	-0.3	-0.3	-1.3	-0.5	-0.6	0.9
AT5G23020 –MAM3	-0.5	-0.3	-0.3	-0.1	0	0	0.2	0.5	1.9
AT4G13770- CYP83A1	-1.3	-1.7	-0.8	-0.6	-0.2	-1.1	0	-0.2	1.3
AT5G61420- MYB28	-0.6	-0.6	-0.4	0	-0.6	0.1	0	0	0.6
AT5G07690- MYB29	-0.2	-0.3	-0.2	-0.2	-0.5	-0.6	0.3	0	0.8
AT1G74090- AtSOT18	-1.1	-0.3	-0.6	0	-0.3	-0.5	0	0.3	1.2
At3g58990- IPMI1	-1.1	-1	-0.6	-0.3	-0.3	-0.9	0.3	0.2	1.5
At2g43100- IPMI2	-1	-1.1	-0.6	-0.3	-0.2	-0.8	-0.5	-0.1	0.9
At4g12030- BAT5	-0.8	-0.5	-0.6	-0.2	-0.3	-1	-0.3	-0.2	1.3
At1g65860-FMO OX1	-0.4	0	-0.3	0	-0.3	-1	-0.2	-0.5	1.6
At1g62540- FMO OX2	0	-0.1	-0.1	0	0	0	-0.2	0.3	1.9
At1g62560- FMO OX3	-0.8	-0.5	-0.5	-0.1	-0.4	-0.7	-0.1	0	1.2
At4g03060- AOP2	-0.4	-0.3	-0.2	-0.1	-0.5	-0.8	0	-0.6	1.7
At4g03050- AOP3	0	0	0	0	-0.1	0	0.1	-0.1	0.6
At1g54540- ESP	0	0	0	0	0	-1.7	-0.2	-0.6	-0.3
At3g14210- ESM1	-0.7	-2.4	-0.5	-0.2	0	0	0.2	0.4	1.6
AT1G16400- CYP79F2	NA	NA	NA	NA	NA	NA	0	0	1.2
AT1G16410 - CYP79F1	NA	NA	NA	NA	NA	NA	-0.4	-0.3	1.2
At1g31180- IPMDH	NA	NA	NA	NA	NA	NA	0.3	0	1.2

Figure 26 Regulation pattern of different aliphatic glucosinolates genes upon treatment with different elicitors and bacteria (NA stands for not available). The color scale represents log2 fold change

Mining of the GENEVESTIGATOR data base (Zimmermann et al., 2004), in respect of the effect of various biotic stresses and pathogenic elicitors on the aliphatic glucosinolates biosynthesis genes, manifested and large similar pattern as was seen for flg22 and OGs in our comparative analysis. The majority of genes either remained unaltered or exhibited reduced expression levels in response to a wide range of biotic stimuli (fig27). This finding further signifies that the induction of the aliphatic glucosinolates cluster of genes is at least not related to various pathogenic treatments and may represent a characteristic signature pattern of *Azospirillum* inoculated plants.

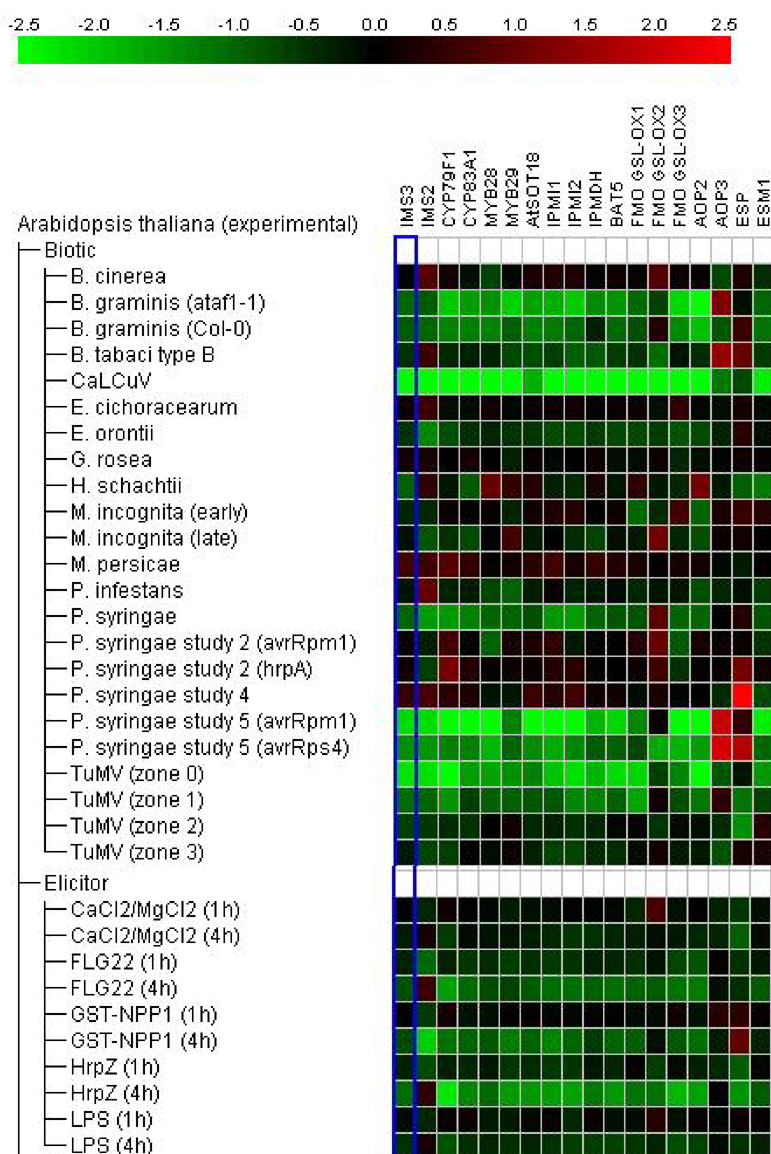


Figure 27 Regulation pattern of different aliphatic glucosinolates genes upon treatment with different elicitors and pathogens

Unlike aliphatic glucosinolates, the trend in case of aromatic and indole glucosinolates manifested altered expression of very few transcripts upon *Azospirillum* treatment. However flg22 and OGs treatment induced quite a few genes pertaining to aromatic and indole glucosinolates biosynthesis.

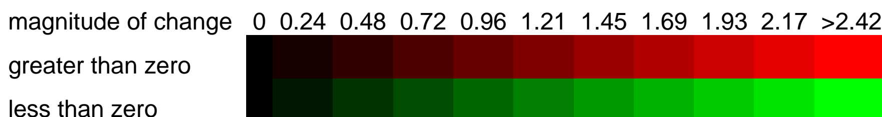
A recent study provided new insights into the role of indolic glucosinolates as potential signaling molecules or coactivators downstream of MAMP-mediated defense responses (Clay et al., 2009).The study shows that biosynthesis of indole glucosinolates, which is induced upon flg22 treatment, is necessary for callose formation. Notably genes involved in the aliphatic glucosinolates were rather down regulated after flg22 treatment in that study. Viewing the exclusive induction of aliphatic glucosinolates genes in our array data and the findings in the above mentioned study, the role of aliphatic glucosinolates as signaling molecules particularly in PGPR-plant interactions cannot be excluded.

4.2.3 Flavonoids

The comparative array analysis revealed another functional cluster of genes, involved in flavonoid biosynthesis and metabolism, with exclusive differential regulation patterns in *Azospirillum* colonized samples. Treatments with flg22 or OGs could neither induce nor repress the pathway. Consistent with the already published reports highlighting the role of different flavonoids compounds in stimulating the colonization of wheat crop by *A.brasilense* (Webster et al., 1998), the induction of flavonoid biosynthesis genes may provide an explanation for the genetic basis of the dynamics of *Azospirillum*-*Arabidopsis* associations. The phenomenon gains importance in view of the fact that successful colonization serves as pre requisite to attain plant growth promotion and other PGPR inoculation related advantages.

By analogy to *Rhizobia*-legume mutualistic association, the role of these flavonoids as inducers of the *Azospirillum* genes enabling them to firmly anchor to plant roots have to be taken into account. This notion gains emphasis particularly in view of the already reported increased flavonoids secretion from

the root exudates of *Phaseolus vulgaris* after inoculation with *A.brasilense* resulting in enhanced nodule numbers (Burdman et al., 1996).



AGI/annotation	Flg22		OGs		C-58		*A.bras.*		
	1hpt	3hpt	1hpt	3hpt	3hpi	6dpi	6hpi	24hpi	96hpi
At4g22880- LDOX	NA	NA	NA	NA	NA	NA	0.6	-0.1	2
At1g03495- AtAAC	NA	NA	NA	NA	NA	NA	-0.2	-0.1	1.4
At5g05270- AtCHI	-0.1	0	-0.1	0.1	-0.3	-0.2	-0.4	0	1.8
At2g22930- UGT79B8	0	0	0	0	-0.3	0	0	-0.2	1.8
At5g54060- UF3GT	0.1	0.1	0.1	0	-0.2	-0.2	-0.5	0.2	2
At5g39050-Transferse	0.7	0.4	0.8	0.4	0.8	0.1	-0.2	-0.2	-1
At4g22870- AtANS	NA	NA	NA	NA	NA	NA	-0.5	0.7	2.4
At5g08640- AtFLS	-0.3	-0.4	-0.1	-0.2	-0.6	-0.2	-0.6	0.1	1.8
At5g42800- DFR	0	0	0	0.1	-0.6	-0.6	0	0.2	1.9
At3g51240- AtF3H	-0.2	-0.3	-0.1	-0.1	-0.7	-0.3	-0.2	0	1.3
At4g25310-Oxidoreductase	NA	NA	NA	NA	NA	NA	0.1	0.3	1.1
At4g35420- DFR1	-0.2	-0.3	-0.1	-0.1	0	-0.7	0	-0.1	-1
At5g13930- CHS-TT4	-0.4	-0.7	-0.2	-0.3	-0.7	-0.3	-0.2	-0.1	1

Figure 28 Regulation trend of different flavonoids related genes upon treatment with different elicitors and bacteria (NA stands for not available). The color scale represents log2 fold change

4.3 *Azospirillum* and plant hormones auxins

The phytohormone auxin, as described in the introductory part of this manuscript, is suggested as the chief agent inducing growth promotion in plants upon *Azospirillum* inoculation. It looks, hence, imperative to screen the genetic basis of different auxin related processes like its synthesis and transport in *Arabidopsis* when associated with the PGPR *Azospirillum brasilense*.

The auxin biosynthetic pathways illustrated so far, still awaits complete and cohesive characterization. The present level of information, however, highlights the existence of both tryptophan (Trp) dependent and independent reactions leading to indole acetic acid (IAA) formation in *Arabidopsis*. The Trp dependent routes include indole-3-acetamide (IAM) pathway, the indole-3-acetaldoxime (IAOx) pathway, the tryptamine (TAM) pathway, and the indole-3-pyruvic acid (IPA) pathway (Woodward and Bartel, 2005).

Transcriptome profiling in our case suggests the indole-3-acetaldoxime (IAOx) pathway indole-3-acetonitrile (IAN) as the main pathway contributing to IAA synthesis in *Arabidopsis* upon PGPR stimulus. The regulation trend of proposed biosynthesis gene (s) active in the other pathways like the IPA and IAM clearly indicates that they are not at least the preferred route for IAA synthesis in the *Arabidopsis-Azospirillum* interaction scenario. This statement attains strength from the repression of indole-3-acetaldehyde (IAAld) specific aldehyde oxidase protein (AAO1) (-0.81 LFC) which catalyze the oxidation of IAAld to form IAA and from the significant down regulation of *Arabidopsis* amidohydrolase (AMI1) which converts IAM to IAA (Pollmann et al., 2003).

Nitrilases enzymes hydrolyzing IAN to IAA are encoded by NIT genes in *Arabidopsis* (Bartling et al., 1992; Bartling et al., 1994). On the basis of their regulation trend in our data, it looks that NIT1 and NIT3 are activated in response to *Azospirillum* inoculation and probably mediates IAN conversion to IAA. Additionally, NIT3 induction has been suggested to relate to lateral root proliferation in *Arabidopsis* (Kutz et al., 2002) which is in agreement with the *Azospirillum* triggered altered plant root morphology.

Auxin controls a number of plant developmental processes which in turn depends on its differential distribution in planta (Benjamins and Scheres, 2008). One of the reasons for this unequal distribution is the underlying transport mechanism operational both at short and long distances. While the former is meant for auxin transport between adjacent cells (polar) the later serves to relocate auxin from the source to roots along the vascular system. This long

distance trafficking is driven by mass flow. Vascular loading and polar transport, however, requires active transporters.

Multiple systems for polar cell-to-cell auxin transport exist in *Arabidopsis*. For instance auxin efflux is thought to be mediated by carrier proteins known as PINFORMED (PIN) proteins and the influx through (ALTERED RESPONSE TO AUXIN AND GRAVITY1 (AUX1) and LIKE AUX (LAX 1, LAX2 & LAX3) family of plasma membrane permeases (Kerr and Bennett, 2007). Still auxin transport also involves members of the *p*-glycoproteins (PGPs) (Geisler and Murphy, 2006).

The array data enabled us to underpin the components of the *Arabidopsis* auxin transport system emanating from the association with *Azospirillum*. Among the PIN family a mixed trend of regulation was observed. The plasma membrane localized PIN1 is expressed in stelar cells and xylem cells in the vascular system and is involved in auxin transportfrom the shoot apex to the root tip (Friml et al., 2002; Galweiler et al., 1998). PIN1 was transcriptionally activated (0.72 LFC) at 96hpi. We also assume that *Arabidopsis* interaction with the PGPR activates the distribution of auxin from stele to the root elongation zone mediated by PIN2 (showing induced transcripts 0, 91LFC in our data), as it has been demonstrated that PIN2 not only controls gravitropism but also regulate the redistribution of auxin to the root elongation zone (Muller et al., 1998). The authors also pinpointed reduced root elongation in the mutants mimicking a phenotype related to high auxins.

Unlike PIN1 and PIN2, down-regulation of PIN3 (-0.64 LFC) indicates that the inoculated plants manage to restrict efflux of auxin from the root hair cells to ensure their improved growth. The suggestion is in agreement with the findings on over expressing lines of PIN3, exhibiting impaired root hair growth (Lee and Cho, 2006). The authors suggested that the over expression gave rise to enhanced efflux of auxin thereby limiting its cytoplasmic levels resulting in inhibition of root hair growth.

Intracellular auxin looks to be further stabilized by the action of significantly higher expression of PIN5 in the treated seedlings. PIN5, which is localized in

endoplasmic reticulum (ER), has been described to function in intracellular auxin homeostasis and metabolism rather than cell to cell transport (Mravec et al., 2009). In addition to auxin homoeostasis within the cell, influx of the hormone might also add to the cellular auxin level as LAX2, an auxin influx carrier, is significantly induced in the *Azospirillum* challenged plants. It would be interesting to investigate whether LAX2 or other auxin influx carriers are involved in transporting the bacterium synthesized auxin into plant cells as is suggested for transport of auxin synthesized by Frankia sp into colonized host cells through AUX1 (Peret et al., 2007).

In the context of plant-*Azospirillum* interaction, this complex interplay of different PINs and LAXs highlights the tight control of auxin transport. How regulation between the efflux and influx carriers during and after the PGPR infection is achieved is still another important unexplored avenue. In Rhizobium-legume interaction, different studies demonstrated flavonoids as regulators of auxin transport (Mathesius et al., 1998; Wasson et al., 2006; Zhang et al., 2009). On the basis of quite a number of flavonoid metabolism related genes differentially regulated upon *Azospirillum* inoculation, we presume their predictive role in modulating auxin transport in the plant in order to achieve a stimulated growth phenotype. Further investigations, on parallel, are needed to elaborate role of auxin not only as an agent of growth and development but also as a signal, facilitating interaction of beneficial bacteria to plants.

Parallel to the effects of plant derived auxin on its growth and development, auxin from the bacterial source should not be over looked as a trigger of improved plant growth . This is particularly important in view of the fact that auxins are quantitatively the most abundant phytohormones secreted by *Azospirillum* species, and are viewed as major factor of plant (root) growth promotion elicited by this bacterium. The improved rooting system is responsible for a better mineral nutrition. At the same time, in a feed-forward loop, it is related to more root exudates resulting in enhanced colonization (Dobbelaere et al., 1999; Steenhoudt and Vanderleyden, 2000). Different factors have been suggested to enhance the synthesis of bacterial derived

auxin. For instance, one model describes root exudates derived auxin as an inducer of the *Azospirillum* indole-3-pyruvate decarboxylase (*ipdc)* gene which ultimately results in increased auxin biosynthesis on the bacterial side (Lambrecht et al., 2000). Noteworthy the *A. brasilense* ipdc promoter is characterized by an auxin-responsive element (AuxRE), claiming similarity to the AuxRE of promoters of plant auxin-inducible genes (Lambrecht et al., 1999). Similarly other reports on legume *Rhizobia* (strain NGR234) interaction pinpoints plant secreted flavonoids as enhancers of bacterial auxin productivity (Prinsen et al., 1991). Detailed investigations would be needed to ascertain the role of such (potential) inducers of the *Azospirillum* auxin biosynthetic genes particularly in face of significant impact of *Azospirillum* inoculation on regulation pattern of *Arabidopsis* genes connected to flavonoids and auxin.

4.4 *Azospirillum* and plant defense responses

Regarding effect of *Azospirillum* treatment on the regulation of other PR proteins, the greatest impact was on the family of plant defensins (PDF), many members of which including PDF1.2 were found with elevated expression. PDFs are small, cysteine-rich, basic proteins possessing antimicrobial activity (Terras et al., 1995). Since activation of defensins is said to be SA independent, one would assume a defense signaling pathway triggered by *Azospirillum* at least not involving SA as a major actor. This is further supported by our finding that SA related genes like SID2, NPR1, TGA and PR1 were unaffected in our treated samples and by a study showing the SA independent nature of disease resistance induced by *Azospirillum* in rice plants (Yasuda et al., 2009). A role of JA and ethylene, however, cannot be excluded. In addition to defensins, a few further identified but functionally uncharacterized PR and PR like proteins might contribute to the defense activation processes triggered by *Azospirillum*. One of the highly induced among them is a PR1 like protein (at4g07820).

Plant metabolites also play an important role in defending plants against various stresses. As discussed elsewhere, aliphatic glucosinolates and flavonoids, besides their role as predicted signaling molecules, appear to be forming the

most striking defense barriers among the metabolites during *Azospirillum* interaction. Similarly, enriched transcripts of genes involved in camalexin and scopoletin synthesis point to the diversified nature of the induction of defense related metabolites related to *Azospirillum* association**.** Significant increase in the transcript levels of an oxidoreductase (at3g13610) in all three time points in the inoculated plants is an example in this regard. This oxidoreductase functions in scopoletin formation in *A. thaliana* (Kai et al., 2008). Scopoletin, a coumarin, is well known for its role in plant defense and originates from the general phenylpropanoid pathway. CYP81F2, a cytochrome P450, has been shown with roles in multiple plant defenses and indole glucosinolate metabolism. A series of publications establish its role in *Arabidopsis* innate immunity responses (Clay et al., 2009), in defense against the green peach aphid (Pfalz et al., 2009) and being part of the processes mediating broad spectrum antifungal defense (Bednarek et al., 2009). Induction of this gene upon *Azospirillum* treatment in all three time points studied, yet explores another component, *Azospirillum* might trigger in *Arabidopsis* to add to the plant defenses.

4.4.1 Induced systemic resistance and the role of jasmonates

In perspective of plant pathogen interactions, the attacked host is not only characterized by initial local defense responses but at the same time exhibits increased defending capability in its distal parts upon subsequent attack by a broad range of pathogens. The strategy adopted by plants is referred to as systemic acquired resistance (SAR) (Durrant and Dong, 2004). Increased SA levels and activation of specific PR proteins are other marker features of SAR. A counterpart of SAR triggered by few specific PGPR strains is termed as induced systemic resistance (ISR) that helps plant to defend against a broad array of diseases (Bakker et al., 2007; van Loon et al., 1998). Thus the PGPR, besides their capability to antagonize rhizospheric pathogens, are able to enhance defenses in the systemic plant tissues upon subsequent attack by pathogens.

Comprehension of signaling pathways operative in ISR has been one of the key objective of current plant-microbe research. In case of ISR, studies dealing with different PGPR and plant species have established that the nature of induced systemic resistance in majority of the cases is SA independent (De Vleesschauwer et al., 2008; Pieterse et al., 1996; Segarra et al., 2009; Yan et al., 2002). However, some PGPR also induce the same immune responses utilizing the SA dependent pathway (Conn et al., 2008; Rudrappa et al., 2008; Tjamos et al., 2005). With the exception of such studies, JA and ET have by and large been described as the key actors in the beneficial microbe triggered defense activation pathways of numerous plant species (Ahn et al., 2007; De Vleesschauwer et al., 2008; Knoester et al., 1999; Pozo et al., 2008; Ryu et al., 2004; Shoresh et al., 2005; Yan et al., 2002).

Consistent with the studies dealing with ISR (Pieterse et al., 1996), we did not observe any accumulation of PR proteins like PR2,PR2, PR5 and other SA related genes. Our transcriptome profiling data, thus, do not suggest any major role for SA in *Azospirillum* mediated plant defenses. Rather the activation of JA associated/marker genes like PDF1.2, LOX2, AOS and few others in essence suggest an important role for jasmonates. Induced levels of transcripts of PDF1.2 and LOX2 were also confirmed by real time PCR. Interestingly quite reasonable repression (-0.52 LFC) of WRKY70 TF, whose constitutive ectopic expression and antisense suppression is respectively related to SA induced PR proteins and JA induced genes (Li et al., 2004), indicates tilt towards JA based signaling. Besides, induction of a JA methyl transferase (JMT) gene (0.85 LFC) which mediates jasmonic acid conversion to the volatile oxylipin methyl jasmonate (MeJA) indicates the probable role of this long distance signaling molecule in the *Azospirillum Arabidopsis* interaction.

4.5 *Azospirillum* and plant root morphology

Azospirillum inoculation prominently affects the root development of different plants ensuring the increased water and mineral acquisition from the soil (Kapulnik et al., 1985a). Viewing the importance, we will primarily discuss the

genetic basis of these changes both in the context of root hair and lateral root development and the role of plant hormone auxin.

4.5.1 *Azospirillum* and plant root hairs

The proliferation of root hairs is one of the most profound effects observed upon inoculation with *Azospirillum* in many plant species. In *Arabidopsis* root hair development can broadly be divided into phases of cell specification, initiation, and elongation. Root hairs arise from epidermal cells. Among the genes controlling root epidermal cell specification, TTG (*TRANSPARENT TESTA/GLABROUS*) or GL2 (*GLABRA2*) function as negative regulators of the differentiation of non hair cells to hair cells (Cho and Cosgrove, 2002). A MYB transcription factor, WER (*WEREWOLF*), is a positive regulates GL2 expression and a mutation in WER is shown to induce root hairs in almost every epidermal root cell (Lee and Schiefelbein, 1999). Besides, *CAPRICE* (CPC), which is a MYB-like protein, functions as a positive regulator for root hair cell differentiation (Wada et al., 1997). *Azospirillum* inoculation seems not only to restrict the differentiation of non hair root cells into hair cells through the action of *GL2* (0.68 log2 fold ; p=0.03) and WER (1.1 log2 fold ; p=0.017) but also ensure the differentiation of root hair cells by slight induction of CPC (0.52LFC: p=0.02). Once the proper hair cells are differentiated, root hair initiation looks to be activated through the action of a basic helix-loop-helix (bHLH) family proteins, RHD6 and AtRSL1 (ROOT HAIR DEFECTIVE 6-LIKE 1) which are specifically required for the development of root hairs and act downstream of the genes that regulate epidermal pattern formation (Menand et al., 2007).

Very recently another BHLH transcription factor called RSL4 (ROOT HAIR DEFECTIVE 6-LIKE 4) is shown to enhance post mitotic root hair cell growth in *Arabidopsis thaliana* (Yi et al., 2010). While loss of RSL4 function in this study gave rise to very short root hairs, the constitutive expression of the same gene resulted in long root hairs. The authors took the view that auxin and low phosphate availability modulate hair cell extension by regulating RSL4 transcript and protein levels. Interestingly RSL4, unlike AtEXPA7 is an immediate

transcriptional target of RHD6 and is expressed only in growing root hairs. Being a transcriptional regulator, RSL4 is predicted to promote the root hair elongation by positively regulating the expression of genes that encode proteins affecting cell growth. Two root hair specific expansins (cell wall–loosening proteins capable of mediating cell wall extension) genes AtEXPA7 & AtEXPA18 with up regulation trend in our data are representative examples in this regard. In nutshell, the inoculated samples in our case exhibited enhanced transcripts levels of gene(s) responsible for all three phases of root hair development, revealing thus the probable genetic basis underlying altered root hair morphology resulting from Arabidopsis / *Azospirillum* interaction.

Root hairs are also implicated in plant microbe interaction. They are shown to mediate the initial contact between the legumes and *Rhizobia* by playing an active part in recognition of bacterial Nod factors and subsequent colonization of the roots by the nitrogen fixing bacteria (Karas et al., 2005). Disruption in the root hair development of the legume plant *Lotus japonicus* results in defective symbiotic interaction, caused by mutation in the *ROOTHAIRLESS1* (LjRHL1) locus. Functional conservation of this locus to the *Arabidopsis* BHLH TFs At2g24260, At4g30980, and At5g58010 was also demonstrated (Karas et al., 2009). These three TFs were shown to function in an at least partially redundant manner to positively regulate root hair development. Owing to significantly higher expression (2.44 LFC) of At4g30980 upon exposure of *Arabidopsis* seedlings to *Azospirillum*, we expect a key role for this protein in plant *Azospirillum* association leading to improved root hair development.

Arabidopsis root hair tip grow requires the production of ROS, (Foreman et al., 2003). *ROOT HAIR DEFECTIVE 2* (RHD2) which encodes respiratory burst oxidase homolog (AtrbohC) is suggested to play a fundamental role in this process. Owing to the presence of calcium binding EF-hands motif and corresponding mutations resulting in greatly hampered ROS production *in vitro* (Takeda et al., 2008) suggests the regulation of RHD2 by cytosolic calcium. In addition, activation of NADPH-xidases requires phosphorylation by kinases Sirichandra FEBS 2009, Ogasawara JBC 2008. *Arabidopsis* plants exposed

to *Azospirillum* infection exhibited enhanced expression of RHD2. This might be one of the factors responsible for improved root hair growth in the treated samples. Furthermore, higher expression levels of *Arabidopsis* CPK2 (0.86 LFC) and CPK4 (0.96 LFC) upon association with *Azospirillum* may correlate with the Ca^{+2} dependent regulation of ROS by RHD2, ensuring the stimulated hair growth.

4.5.2 *Azospirillum* and plant lateral root morphology

Azospirillum is well known for its effect on lateral root development in many plant species. However, the plant genetic components involved still need to be uncovered. The microarray analysis results in question, present the first comprehensive but preliminary view of these missing links.

The regulation trend of *Arabidopsis* genes after being treated with *Azospirillum* revealed different proteins functioning in improved lateral root status.

4.5.2.1 Nitrate transporters

Plants respond to nitrogen limiting conditions, by inducing the high affinity transport systems (HATS) for NO_3^- or NH_4^+ uptake and by stimulation of lateral root growth, thus enabling the plants to have better access to N sources. Plants treated with *Azospirillum* significantly induced the expression levels of at least four members of the high affinity nitrate transport family proteins (NRT2.1, NRT2.2, NRT2.4 & NRT3.1). In view of adequate nitrate supply in the growth media, the differential regulation pattern appears unusual. However, it does imply that inoculation with PGPR probably interfere with the regulatory mechanisms driving NO_3^- dependent root development as suggested for the PGPR *Phyllobacterium sp* in its interaction with *Arabidopsis* (Mantelin et al., 2006). Although high $NO3^-$ supply has been predicted to negatively regulate lateral root elongation (Zhang et al., 1999), reports exist signifying HA nitrate transporter NRT2.1 having a direct stimulatory role in initiation of LR primordia (Mantelin et al., 2006) consistent with the role of *Azospirillum* positively affecting

lateral root development (Fallik et al., 1994; Kapulnik et al., 1987). Unlike HA nitrate transporters, no significant induction of Ammonium transporters (AMT) were observed in the inoculated seedlings, AMT1.3 being the only transcript exhibiting an induced state (0.80LFC).

Further genetic components functioning in stimulating lateral root growth were revealed upon Azospirillum infection. It looks that exposure to PGPR not only act to promote the activity of positive lateral root regulators but also represses the expression of genes which negatively regulate lateral root development. An AP2/B3 domain transcription factor Rav1 (see results part) and WRKY 75 are examples in this regard. The WRKY75 TF, known to negatively regulate lateral root length and root hair number (Devaiah et al., 2007).

5 Literature cited

Abrahams, S., Lee, E., Walker, A.R., Tanner, G.J., Larkin, P.J., and Ashton, A.R. (2003). The Arabidopsis TDS4 gene encodes leucoanthocyanidin dioxygenase (LDOX) and is essential for proanthocyanidin synthesis and vacuole development. Plant J 35, 624-636.

Ahn, I.P., Lee, S.W., and Suh, S.C. (2007). Rhizobacteria-induced priming in Arabidopsis is dependent on ethylene, jasmonic acid, and NPR1. Mol Plant Microbe Interact 20, 759-768.

Ahn, Y.O., Shimizu, B., Sakata, K., Gantulga, D., Zhou, Z., Bevan, D.R., and Esen, A. (2010). Scopolin-hydrolyzing beta-glucosidases in roots of Arabidopsis. Plant Cell Physiol 51, 132-143.

Akiyama, K., Matsuzaki, K., and Hayashi, H. (2005). Plant sesquiterpenes induce hyphal branching in arbuscular mycorrhizal fungi. Nature 435, 824-827.

Amooaghaie, R., Mostajeran, A., and Emtiazi, G. (2002). The effect of compatible and incompatible *Azospirillum brasilense* strains on proton efflux of intact wheat roots–Azospirillum and proton efflux of wheat root. . Plant Soil 243, 155-160.

Antonyuk, L., Fomina, O., Kalinina, A., Semenov, S., Nesmeyanova, and M. Ignatov, V. (1995). Wheat lectin possibly serves as a signal molecule in the Azospirillum-wheat association. In: Azospirillum VI and Related Microorganisms (Fendrik, I, Del Gallo,M, Vanderleyden, J and de Zamaroczy, M, Eds), pp. 319-325.Springer, Berlin.

Antonyuk, L.P., Fomina, O.R., Galkin, M.A., and Ignatov, V.V. (1993). The e¡ect of wheat germ agglutinin on dinitrogen ¢xation, glutamine synthetase activity and ammonia excretion in *Azospirillum brasilense* Sp245. FEMS Microbiol Lett 110, 285-290.

Antonyuk, L.P., Sumaroka, M.V., and Ignatov, V.V. (1999). Effect of wheat lectin on the membrane phospholipid content of *Azospirillum brasilense*. Appl Biochem Microbiol 35, 53–55.

Arsovski, A.A., Popma, T.M., Haughn, G.W., Carpita, N.C., McCann, M.C., and Western, T.L. (2009). AtBXL1 encodes a bifunctional beta-D-xylosidase/alpha-L-arabinofuranosidase required for pectic arabinan modification in Arabidopsis mucilage secretory cells. Plant Physiol 150, 1219-1234.

Asai, T., Tena, G., Plotnikova, J., Willmann, M.R., Chiu, W.L., Gomez-Gomez, L., Boller, T., Ausubel, F.M., and Sheen, J. (2002). MAP kinase signalling cascade in Arabidopsis innate immunity. Nature 415, 977-983.

Assmus, B., Hutzler, P., Kirchhof, G., Amann, R., Lawrence, J.R., and Hartmann, A. (1995). In Situ Localization of *Azospirillum brasilense* in the Rhizosphere of Wheat with Fluorescently Labeled, rRNA-Targeted Oligonucleotide Probes and Scanning Confocal Laser Microscopy. Appl Environ Microbiol 61, 1013-1019.

Atkinson, M.M., Huang, J.S., and Knopp, J.A. (1985). The Hypersensitive Reaction of Tobacco to *Pseudomonas syringae* pv. pisi: Activation of a Plasmalemma K/H Exchange Mechanism. Plant Physiol 79, 843-847.

B´ecard, G., and Pich´e, Y. (1989). Fungal growth stimulation by CO2 and root exudates in vesicular-arbuscular mycorrhizal symbiosis. Appl Environ Microbiol 55, 2320–2325.

Bachhawat, A.K., and Ghosh, S. (1987). Iron transport in *Azospirillum brasilense*, role of the siderophores spirilobactin. J Gen Microbiol, 1759-1765.

Bakker, P.A., Pieterse, C.M., and van Loon, L.C. (2007). Induced Systemic Resistance by Fluorescent Pseudomonas spp. Phytopathology 97, 239-243.

Barassi, C.A., Ayrault, G., Creus, C.M., Sueldo, R.J., and Sobrero, M.T. (2006). Seed inoculation with Azospirillum mitigates NaCl effects on lettuce. Scientia Horticulturae 109, 8-14.

Bartling, D., Seedorf, M., Mithofer, A., and Weiler, E.W. (1992). Cloning and expression of an Arabidopsis nitrilase which can convert indole-3-acetonitrile to the plant hormone, indole-3-acetic acid. Eur J Biochem 205, 417-424.

Bartling, D., Seedorf, M., Schmidt, R.C., and Weiler, E.W. (1994). Molecular characterization of two cloned nitrilases from *Arabidopsis thaliana*: key enzymes in biosynthesis of the plant hormone indole-3-acetic acid. Proc Natl Acad Sci U S A 91, 6021-6025.

Bashan, Y., and De-Bashan, L.E. (2002). Protection of tomato seedlings against infection by *Pseudomonas syringae* pv. tomato by using the plant growth-promoting bacterium *Azospirillum brasilense*. Appl Environ Microbiol 68, 2637-2643.

Bashan, Y., Holguin, G., and de-Bashan, L.E. (2004). Azospirillum-plant relationships: physiological, molecular, agricultural, and environmental advances (1997-2003). Can J Microbiol 50, 521-577.

Bauer, Z., Gomez-Gomez, L., Boller, T., and Felix, G. (2001). Sensitivity of different ecotypes and mutants of *Arabidopsis thaliana* toward the bacterial elicitor flagellin correlates with the presence of receptor-binding sites. J Biol Chem 276, 45669-45676.

Becker, D., Stanke, R., Fendrik, I., Frommer, W.B., Vanderleyden, J., Kaiser, W.M., and Hedrich, R. (2002). Expression of the NH(+)(4)-transporter gene LEAMT1;2 is induced in tomato roots upon association with N(2)-fixing bacteria. Planta 215, 424-429.

Becking, J.H. (1963). Fixation of molecular nitrogen by an aerobic vibrio or spirillum. J Microbiol Serol 29, 326.

Bednarek, P., Pislewska-Bednarek, M., Svatos, A., Schneider, B., Doubsky, J., Mansurova, M., Humphry, M., Consonni, C., Panstruga, R., Sanchez-Vallet, A., et al. (2009). A glucosinolate metabolism pathway in living plant cells mediates broad-spectrum antifungal defense. Science 323, 101-106.

Beijerinck, M.W. (1925). Über ein Spirillum, welches freien Stickstoff binden kann? Zentralblatt für Bakteriologie Parasitenkunde, Infektionskrankheiten and Hygiene Naturewissenschaftliche Abt II 63, 353–359.

Benizri, E., Baudoin, E., and Guckert, A. (2001). Root colonization by inoculated plant growth-promoting rhizobacteria. Biocontrol Sci Technol 11, 557–574.

Benjamins, R., and Scheres, B. (2008). Auxin: the looping star in plant development. Annu Rev Plant Biol 59, 443-465.

Benson, D.R., and Silvester, W.B. (1993). Biology of Frankia strains, actinomycete symbionts of actinorhizal plants. Microbiol Rev 57, 293-319.

Bernhardt, C., and Tierney, M.L. (2000). Expression of AtPRP3, a proline-rich structural cell wall protein from Arabidopsis, is regulated by cell-type-specific developmental pathways involved in root hair formation. Plant Physiol 122, 705-714.

Blaha, D., Prigent-Combaret, C., Mirza, M.S., and Moenne-Loccoz, Y. (2006). Phylogeny of the 1-aminocyclopropane-1-carboxylic acid deaminase-encoding gene acdS in phytobeneficial and pathogenic Proteobacteria and relation with strain biogeography. FEMS Microbiol Ecol 56, 455-470.

Boller, T., and Felix, G. (2009). A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. Annu Rev Plant Biol 60, 379-406.

Boller, T., Felix, G., Baureithel, K., Fath, A., and Regenass, M. (1995). Chemoperception of Microbial Signals and of Systemin. J Cell Biochem, 506-506.

Bolwell, G.P. (1999). Role of active oxygen species and NO in plant defence responses. Curr Opin Plant Biol 2, 287-294.

Bonfante, P., and Genre, A. (2008). Plants and arbuscular mycorrhizal fungi: an evolutionary-developmental perspective. Trends Plant Sci 13, 492-498.

Bottini, R., Fulchieri, M., Pearce, D., and Pharis, R.P. (1989). Identification of Gibberellins A(1), A(3), and Iso-A(3) in Cultures of *Azospirillum lipoferum*. Plant Physiol 90, 45-47.

Buee, M., Rossignol, M., Jauneau, A., Ranjeva, R., and Becard, G. (2000). The pre-symbiotic growth of arbuscular mycorrhizal fungi is induced by a branching factor partially purified from plant root exudates. Mol Plant Microbe Interact 13, 693-698.

Burdman, S., Dulguerova, G., Okon, Y., and Jurkevitch, E. (2001). Purification of the major outer membrane protein of *Azospirillum brasilense*, its affinity to plant roots, and its involvement in cell aggregation. Mol Plant Microbe Interact 14, 555-561.

Burdman, S., Jurkevitch, E., Schwartsburd, B., and Okon, Y. (1999). Involvement of outer-membrane proteins in the aggregation of *Azospirillum brasilense*. Microbiology 145 (Pt 5), 1145-1152.

Burdman, S., Volpin, H., Kigel, J., Kapulnik, Y., and Okon, Y. (1996). Promotion of nod Gene Inducers and Nodulation in Common Bean (Phaseolus vulgaris) Roots Inoculated with *Azospirillum brasilense* Cd. Appl Environ Microbiol 62, 3030-3033.

Burleigh, S.H., Kristensen, B.K., and Bechmann, I.E. (2003). A plasma membrane zinc transporter from *Medicago truncatula* is up-regulated in roots by Zn fertilization, yet down-regulated by arbuscular mycorrhizal colonization. Plant Mol Biol 52, 1077-1088.

Camehl, I., Sherameti, I., Venus, Y., Bethke, G., Varma, A., Lee, J., and Oelmuller, R. (2010). Ethylene signalling and ethylene-targeted transcription factors are required to balance beneficial and nonbeneficial traits in the symbiosis between the endophytic fungus Piriformospora indica and *Arabidopsis thaliana*. New Phytol.

Cappellazzo, G., Lanfranco, L., Fitz, M., Wipf, D., and Bonfante, P. (2008). Characterization of an amino acid permease from the endomycorrhizal fungus Glomus mosseae. Plant Physiol 147, 429-437.

Cardenas, L., Martinez, A., Sanchez, F., and Quinto, C. (2008). Fast, transient and specific intracellular ROS changes in living root hair cells responding to Nod factors (NFs). Plant J 56, 802-813.

Carrillo, A.E., Li, C.Y., and Bashan, Y. (2002). Increased acidification in the rhizosphere of cactus seedlings induced by *Azospirillum brasilense*. Naturwissenschaften 89, 428-432.

Casanovas, E.M., Barassi, C.A., and R.J., S. (2002). Azospirillum inoculation mitigates water stress effects in maize seedlings. Cereal Research Communications 30, 343-350.

Castellanos, T., Ascencio, F., and Bashan, Y. (1998). Cell-surface lectins of Azospirillum spp. Curr Microbiol 36, 241-244.

Chabaud M, Venard C, Defaux-Petras A, Becard G, and DG., B. (2002). Targeted inoculation of *Medicago truncatula* in vitro root cultures reveals MtENOD11 expression during early stages of infection by arbuscular mycorrhizal fungi. New Phytol 156, 265–273.

Chen, K., Fan, B., Du, L., and Chen, Z. (2004). Activation of hypersensitive cell death by pathogen-induced receptor-like protein kinases from Arabidopsis. Plant Mol Biol 56, 271-283.

Cho, H.T., and Cosgrove, D.J. (2002). Regulation of root hair initiation and expansin gene expression in Arabidopsis. Plant Cell 14, 3237-3253.

Clay, N.K., Adio, A.M., Denoux, C., Jander, G., and Ausubel, F.M. (2009). Glucosinolate metabolites required for an Arabidopsis innate immune response. Science 323, 95-101.

Cohen, A.C., Bottini, R., and Piccoli, P.N. (2008). *Azospirillum brasilense* Sp 245 produces ABA in chemically-defined culture medium and increases ABA content in arabidopsis plants. Plant Growth Regul 54, 97-103.

Cohen, M.F., Sakihama, Y., Takagi, Y.C., Ichiba, T., and Yamasaki, H. (2002). Synergistic effect of deoxyanthocyanins from symbiotic fern Azolla spp. on hrmA gene induction in the cyanobacterium Nostoc punctiforme. Mol Plant Microbe Interact 15, 875-882.

Conn, V.M., Walker, A.R., and Franco, C.M. (2008). Endophytic actinobacteria induce defense pathways in *Arabidopsis thaliana*. Mol Plant Microbe Interact 21, 208-218.

Cordier, C., Pozo MJ, Barea JM, Gianinazzi S, and V, G.-P. (1998). Cell defence responses associated with localized and systemic resistance to Phytophthora parasitica induced in tomato by an arbuscular mycorrhizal fungus. Molecular Plant-Microbe Interactions 11, 1017-1028.

Costacurta, A., Keijers, V., and Vanderleyden, J. (1994). Molecular cloning and sequence analysis of an *Azospirillum brasilense* indole-3-pyruvate decarboxylase gene. Mol Gen Genet 243, 463-472.

Costacurta, A., and Vanderleyden, J. (1995). Synthesis of phytohormones by plant-associated bacteria. Crit Rev Microbiol 21, 1-18.

Creus, C.M., Graziano, M., Casanovas, E.M., Pereyra, M.A., Simontacchi, M., Puntarulo, S., Barassi, C.A., and Lamattina, L. (2005). Nitric oxide is involved in the *Azospirillum brasilense*-induced lateral root formation in tomato. Planta 221, 297-303.

Creus, C.M., Sueldo, R.J., and Barassi, C.A. (1998). Water relations in Azospirillum -inoculated wheat seedlings under osmotic stress. Can J Bot 76, 238-244.

Dakora, F.D., and Phillips, D.A. (1996). Diverse functions of isoflavonoids in legumes transcend anti-microbial definitions of phytoalexins. Physiol Mol Plant P 49, 1-20.

Dangl, J.L., and Jones, J.D. (2001). Plant pathogens and integrated defence responses to infection. Nature 411, 826-833.

Dardanelli, M.S., Fernández de Córdoba, F.J., Rosario Espuny, M., Rodríguez Carvajal, M.A., Soria Díaz, M.E., Gil Serrano, A.M., Okon, Y., and Megías, M. (2008). Effect of *Azospirillum brasilense* coinoculated with Rhizobium on Phaseolus vulgaris flavonoids and Nod factor production under salt stress. Soil Biol Biochem 40, 2713–2721.

De Vleesschauwer, D., Djavaheri, M., Bakker, P.A., and Hofte, M. (2008). *Pseudomonas fluorescens* WCS374r-induced systemic resistance in rice against Magnaporthe oryzae is based on pseudobactin-mediated priming for a salicylic acid-repressible multifaceted defense response. Plant Physiol 148, 1996-2012.

Del Gallo, M., Negi, M., and Neyra, C.A. (1989). Calcofluor- and lectin-binding exocellular polysaccharides of *Azospirillum brasilense* and *Azospirillum lipoferum*. J Bacteriol 171, 3504-3510.

den Hartog, M., Musgrave, A., and Munnik, T. (2001). Nod factor-induced phosphatidic acid and diacylglycerol pyrophosphate formation: a role for phospholipase C and D in root hair deformation. Plant J 25, 55-65.

Denoux, C., Galletti, R., Mammarella, N., Gopalan, S., Werck, D., De Lorenzo, G., Ferrari, S., Ausubel, F.M., and Dewdney, J. (2008). Activation of defense response pathways by OGs and Flg22 elicitors in Arabidopsis seedlings. Mol Plant 1, 423-445.

Devaiah, B.N., Karthikeyan, A.S., and Raghothama, K.G. (2007). WRKY75 transcription factor is a modulator of phosphate acquisition and root development in Arabidopsis. Plant Physiol 143, 1789-1801.

Divol, F., Vilaine, F., Thibivilliers, S., Kusiak, C., Sauge, M.H., and Dinant, S. (2007). Involvement of the xyloglucan endotransglycosylase/hydrolases encoded by celery XTH1 and Arabidopsis XTH33 in the phloem response to aphids. Plant Cell Environ 30, 187-201.

Dixon, D.P., Davis, B.G., and Edwards, R. (2002). Functional divergence in the glutathione transferase superfamily in plants. Identification of two classes with putative functions in redox homeostasis in *Arabidopsis thaliana*. J Biol Chem 277, 30859-30869.

Dobbelaere, S., Croonenborghs, A., Thys, A., Ptacek, D., Vanderleyden, J., and Dutto, P., et al (2001). Responses of agronomically important crops to inoculation with Azospirillum. Aust J Plant Physiol 28, 871-879.

Dobbelaere, S., Croonenborghs, A., Thys, A., Vande Broek, A., and Vanderleyden, J. (1999). Phytostimulatory effect of *Azospirillum brasilense* wild type and mutant strains altered in IAA production on wheat. Plant Soil 212, 155-164.

Dobereiner, J., and Baldani, V.L. (1979). Selective infection of maize roots by streptomycin-resistant *Azospirillum lipoferum* and other bacteria. Can J Microbiol 25, 1264-1269.

Döbereiner, J., and Day, J.M. (1976). Associative symbioses in tropical grasses: Characterization of microorganisms and dinitrogen fixing sites. In: Newton, W E and C JNyman (Eds) Proceedings of the First International Symposium on Nitrogen Fixation Washington State University Press Pullman, WA 2, 518-538.

Durrant, W.E., and Dong, X. (2004). Systemic acquired resistance. Annu Rev Phytopathol 42, 185-209.

Eckert, B., Weber, O.B., Kirchhof, G., Halbritter, A., Stoffels, M., and Hartmann, A. (2001). *Azospirillum doebereinerae* sp. nov., a nitrogen-fixing bacterium associated with the C4-grass Miscanthus. Int J Syst Evol Microbiol 51, 17-26.

Ehrhardt, D.W., Atkinson, E.M., and Long, S.R. (1992). Depolarization of alfalfa root hair membrane potential by *Rhizobium meliloti* Nod factors. Science 256, 998-1000.

El-Khawas, H., and Adachi, K. (1999). Identification and quantification of auxins in culture media of Azospirillum and Klebsiella and their effect on rice roots. Biol Fertil Soils 28, 377-381.

Escamilla-Trevino, L.L., Chen, W., Card, M.L., Shih, M.C., Cheng, C.L., and Poulton, J.E. (2006). *Arabidopsis thaliana* beta-Glucosidases BGLU45 and BGLU46 hydrolyse monolignol glucosides. Phytochemistry 67, 1651-1660.

Fallik, E., Sarig, S., and Okon, Y. (1994). Morphology and physiology of plant roots associated with Azospirillum. CRC Press, Boca Raton, FL, USA, 77-86.

Felix, G., Duran, J.D., Volko, S., and Boller, T. (1999). Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. Plant J 18, 265-276.

Felle, H.H., Kondorosi, E., Kondorosi, A., and Schultze, M. (1996). Rapid alkalinization in alfalfa root hairs in response to rhizobial lipochitooligosaccharide signals. Plant J 10, 295-301.

Felle, H.H., Kondorosi, E., Kondorosi, A., and Schultze, M. (1999). Elevation of the cytosolic free [Ca2+] is indispensable for the transduction of the Nod factor signal in alfalfa. Plant Physiol 121, 273-280.

Felle, H.H., Kondorosi, E., Kondorosi, A., and Schultze, M. (2000). How alfalfa root hairs discriminate between Nod factors and oligochitin elicitors. Plant Physiol 124, 1373-1380.

Field, B., and Osbourn, A.E. (2008). Metabolic diversification--independent assembly of operon-like gene clusters in different plants. Science 320, 543-547.

Foreman, J., Demidchik, V., Bothwell, J.H., Mylona, P., Miedema, H., Torres, M.A., Linstead, P., Costa, S., Brownlee, C., Jones, J.D., et al. (2003). Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. Nature 422, 442-446.

Friml, J., Benkova, E., Blilou, I., Wisniewska, J., Hamann, T., Ljung, K., Woody, S., Sandberg, G., Scheres, B., Jurgens, G., et al. (2002). AtPIN4 mediates sink-driven auxin gradients and root patterning in Arabidopsis. Cell 108, 661-673.

Gahoonia, T.S., and Nielsen, N.E. (1998). Direct evidence on participation of root hairs in phosphorus (P-32) uptake from soil. Plant Soil 198, 147-152.

Gahoonia, T.S., Nielsen, N.E., Joshi, P.A., and Jahoor, A. (2001). A root hairless barley mutant for elucidating genetic of root hairs and phosphorus uptake. Plant Soil 235, 211-219.

Gallori, E., and Bazzicalupo, M. (1985). Effect of nitrogen compounds on nitrogenase activity in *Azospirillum brasilense*. . FEMS Microbiol Lett 28, 35-38.

Galweiler, L., Guan, C., Muller, A., Wisman, E., Mendgen, K., Yephremov, A., and Palme, K. (1998). Regulation of polar auxin transport by AtPIN1 in Arabidopsis vascular tissue. Science 282, 2226-2230.

Geisler, M., and Murphy, A.S. (2006). The ABC of auxin transport: the role of p-glycoproteins in plant development. FEBS Lett 580, 1094-1102.

German, M.A., Burdman, S., Okon, Y., and Kigel, J. (2000). Effects of *Azospirillum brasilense* on root morphology of common bean (Phaseolus vulgaris L.) under different water regimes. Biol Fertil Soils 32:, 259-264.

Geurts, R., and Bisseling, T. (2002). Rhizobium nod factor perception and signalling. Plant Cell 14 Suppl, S239-249.

Gianinazzi-Pearson V, Branzanti B, and S., G. (1989). In vitro enhancement of spore germination and early hyphal growth of a vesicular-arbuscular mycorrhizal fungus by host root exudates and plant flavonoids. Symbiosis 7, 243-255.

Gianinazzi-Pearson, V., and Denarie, J. (1997). Red carpet genetic programmes for root endosymbioses. Trends in Plant Science 2, 371-372.

Gibson, K.E., Kobayashi, H., and Walker, G.C. (2008). Molecular determinants of a symbiotic chronic infection. Annu Rev Genet 42, 413-441.

Gigolashvili, T., Yatusevich, R., Rollwitz, I., Humphry, M., Gershenzon, J., and Flugge, U.I. (2009). The plastidic bile acid transporter 5 is required for the biosynthesis of methionine-derived glucosinolates in *Arabidopsis thaliana*. Plant Cell 21, 1813-1829.

Giovannetti M, Sbrana C, Avio L, Citernesi AS, and C., L. (1993). Differential hyphal morphogenesis in arbuscular mycorrhizal fungi during pre-infection stages. New Phytol 125, 587-593.

Glick, B.R. (1995). The enhancement of plant growth by free-living bacteria. Can J Microbiol 41, 109-117.

Goldstein, I.J., Hughes, R.C., Monsigny, M., Osawa, T., and Sharon, N. (1980). What should be called a lectin? Nature 66, 285-291.

Gomez-Gomez, L., and Boller, T. (2000). FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in Arabidopsis. Mol Cell 5, 1003-1011.

Gomez-Gomez, L., Felix, G., and Boller, T. (1999). A single locus determines sensitivity to bacterial flagellin in *Arabidopsis thaliana*. Plant J 18, 277-284.

Gonzalez, L.E., and Bashan, Y. (2000). Increased growth of the microalga Chlorella vulgaris when coimmobilized and cocultured in alginate beads with the plant-growth-promoting bacterium *Azospirillum brasilense*. Appl Environ Microbiol 66, 1527-1531.

Grant, J.J., Yun, B.W., and Loake, G.J. (2000). Oxidative burst and cognate redox signalling reported by luciferase imaging: identification of a signal network that functions independently of ethylene, SA and Me-JA but is dependent on MAPKK activity. Plant J 24, 569-582.

Guether, M., Balestrini, R., Hannah, M., He, J., Udvardi, M.K., and Bonfante, P. (2009a). Genome-wide reprogramming of regulatory networks, transport, cell wall and membrane biogenesis during arbuscular mycorrhizal symbiosis in Lotus japonicus. New Phytol 182, 200-212.

Guether, M., Neuhauser, B., Balestrini, R., Dynowski, M., Ludewig, U., and Bonfante, P. (2009b). A mycorrhizal-specific ammonium transporter from Lotus japonicus acquires nitrogen released by arbuscular mycorrhizal fungi. Plant Physiol 150, 73-83.

Hadas, R., and Okon, Y. (1987). Effect of *Azospirillum brasilense* inoculation on root morphology and respiration in tomato seedlings. Biol FertilSoils 5, 241-247.

Hahlbrock, K., and Scheel, D. (1989). Physiology and molecular biology of phenypropanoid metabolism. Annual Review of Plant Physiology of Plant Molecular Biology 40, 347-369.

Hall, P.G., and Krieg, N.R. (1983). Swarming of *Azospirillum brasilense* on solid media. Can J Microbiol 29, 1592-1594.

Hamaoui, B., Abbadi, J.M., Burdman, S., Rashid, A., Sarig, S., and Okon, Y. (2001). Effects of inoculation with *Azospirillum brasilense* on chickpeas (Cicer arietinum) and faba beans (Vicia faba) under different growth conditions. Agronomie 21, 553-560.

Harari, A., Kigel, J., and Okon, Y. (1988). Involvement of IAA in the interaction between *Azospirillum brasilense* and Panicum miliaceum roots. Plant Soil 110, 275-282.

Harrison, M.J. (2005). Signaling in the arbuscular mycorrhizal symbiosis. Annu Rev Microbiol 59, 19-42.

Harrison, M.J., and van Buuren, M.L. (1995). A phosphate transporter from the mycorrhizal fungus Glomus versiforme. Nature 378, 626-629.

Hecht-Buchholz, C. (1998). The apoplast-habitat of endophytic dinitrogen-fixing bacteria and their significance for the nitrogen nutrition of nonleguminous plants. Z PflanzenernaehrBodenkd 161, 509–520.

Heim, M.A., Jakoby, M., Werber, M., Martin, C., Weisshaar, B., and Bailey, P.C. (2003). The basic helix-loop-helix transcription factor family in plants: a genome-wide study of protein structure and functional diversity. Mol Biol Evol 20, 735-747.

Holguin, G., and Glick, B.R. (2003). Transformation of *Azospirillum brasilense* Cd with an ACC deaminase gene from enterobacter cloacae UW4 fused to the Tet r gene promoter improves its fitness and plant growth promoting ability. Microb Ecol 46, 122-133.

Hruba, P., Honys, D., Twell, D., Capkova, V., and Tupy, J. (2005). Expression of beta-galactosidase and beta-xylosidase genes during microspore and pollen development. Planta 220, 931-940.

Hu, Y.X., Wang, Y.X., Liu, X.F., and Li, J.Y. (2004). Arabidopsis RAV1 is down-regulated by brassinosteroid and may act as a negative regulator during plant development. Cell Res 14, 8-15.

Idriss, E.E., Makarewicz, O., Farouk, A., Rosner, K., Greiner, R., Bochow, H., Richter, T., and Borriss, R. (2002). Extracellular phytase activity of Bacillus amyloliquefaciens FZB45 contributes to its plant-growth-promoting effect. Microbiology 148, 2097-2109.

Imaizumi-Anraku, H., Takeda, N., Charpentier, M., Perry, J., Miwa, H., Umehara, Y., Kouchi, H., Murakami, Y., Mulder, L., Vickers, K., et al. (2005). Plastid proteins crucial for symbiotic fungal and bacterial entry into plant roots. Nature 433, 527-531.

Jacobsen, C.S. (1997). Plant protection and rhizosphere colonization of barley by seed inoculated herbicide degrading Burkholderia (Pseudomonas) cepacia DBO1(pRO101) in 2,4-D contaminated soil. . Plant Soil 189, 139-144.

Jain, D.K., and Patriquin, D.G. (1985). Characterization of a substance produced by Azospirillum which causes branching of wheat root hairs. Can J Microbiol 31, 206-210.

Javot, H., Penmetsa, R.V., Terzaghi, N., Cook, D.R., and Harrison, M.J. (2007). A *Medicago truncatula* phosphate transporter indispensable for the arbuscular mycorrhizal symbiosis. Proc Natl Acad Sci U S A 104, 1720-1725.

Jiang, Y., Yang, B., Harris, N.S., and Deyholos, M.K. (2007). Comparative proteomic analysis of NaCl stress-responsive proteins in Arabidopsis roots. J Exp Bot 58, 3591-3607.

Jofuku, K.D., den Boer, B.G., Van Montagu, M., and Okamuro, J.K. (1994). Control of Arabidopsis flower and seed development by the homeotic gene APETALA2. Plant Cell 6, 1211-1225.

Jones, J.D., and Dangl, J.L. (2006). The plant immune system. Nature 444, 323-329.

Jourdan, E., Henry, G., Duby, F., Dommes, J., Barthelemy, J.P., Thonart, P., and Ongena, M. (2009). Insights into the defense-related events occurring in plant cells following perception of surfactin-type lipopeptide from *Bacillus subtilis*. Mol Plant Microbe Interact 22, 456-468.

Journet, E.P., El-Gachtouli, N., Vernoud, V., de Billy, F., Pichon, M., Dedieu, A., Arnould, C., Morandi, D., Barker, D.G., and Gianinazzi-Pearson, V. (2001). *Medicago truncatula* ENOD11: a novel RPRP-encoding early nodulin gene expressed during mycorrhization in arbuscule-containing cells. Mol Plant Microbe Interact 14, 737-748.

Juge, N., Mithen, R.F., and Traka, M. (2007). Molecular basis for chemoprevention by sulforaphane: a comprehensive review. Cell Mol Life Sci 64, 1105-1127.

Kai, K., Mizutani, M., Kawamura, N., Yamamoto, R., Tamai, M., Yamaguchi, H., Sakata, K., and Shimizu, B. (2008). Scopoletin is biosynthesized via ortho-hydroxylation of feruloyl CoA by a 2-oxoglutarate-dependent dioxygenase in *Arabidopsis thaliana*. Plant J 55, 989-999.

Kamiya, T., Tanaka, M., Mitani, N., Ma, J.F., Maeshima, M., and Fujiwara, T. (2009). NIP1;1, an aquaporin homolog, determines the arsenite sensitivity of *Arabidopsis thaliana*. J Biol Chem 284, 2114-2120.

Kaneko, T., Minamisawa, K., Isawa, T., Nakatsukasa, H., Mitsui, H., Kawaharada, Y., Nakamura, Y., Watanabe, A., Kawashima, K., Ono, A., et al. (2010). Complete genomic structure of the cultivated rice endophyte Azospirillum sp. B510. DNA Res 17, 37-50.

Kapulnik, Y., Gafny, R., and Okon, Y. (1985a). Effect of Azospirillum Spp Inoculation on Root Development and No3- Uptake in Wheat (Triticum-Aestivum Cv Miriam) in Hydroponic Systems. Can J Bot 63, 627-631.

Kapulnik, Y., Okon, Y., and Henis, Y. (1985b). Changes in root morphology of wheat caused by Azospirillum inoculation. CanJ Bot 31, 881-887.

Kapulnik, Y., Okon, Y., and Henis, Y. (1987). Yield response of spring wheat cultivars (Triticum aestivum and T. turgidum) to inoculation with *Azospirillum brasilense* under field conditions BiolFertilSoils 4, 27-35.

Karas, B., Amyot, L., Johansen, C., Sato, S., Tabata, S., Kawaguchi, M., and Szczyglowski, K. (2009). Conservation of lotus and Arabidopsis basic helix-loop-helix proteins reveals new players in root hair development. Plant Physiol 151, 1175-1185.

Karas, B., Murray, J., Gorzelak, M., Smith, A., Sato, S., Tabata, S., and Szczyglowski, K. (2005). Invasion of Lotus japonicus root hairless 1 by Mesorhizobium loti involves the nodulation factor-dependent induction of root hairs. Plant Physiol 137, 1331-1344.

Karpati, E., Kiss, P., Ponyi, T., Fendrik, I., de Zamaroczy, M., and Orosz, L. (1999). Interaction of *Azospirillum lipoferum* with wheat germ agglutinin stimulates nitrogen fixation. J Bacteriol 181, 3949-3955.

Kerr, I.D., and Bennett, M.J. (2007). New insight into the biochemical mechanisms regulating auxin transport in plants. Biochem J 401, 613-622.

Khammas, K.M., Ageron, E., Grimont, P.A., and Kaiser, P. (1989). *Azospirillum irakense* sp. nov., a nitrogen-fixing bacterium associated with rice roots and rhizosphere soil. Res Microbiol 140, 679-693.

Kistner, C., Winzer, T., Pitzschke, A., Mulder, L., Sato, S., Kaneko, T., Tabata, S., Sandal, N., Stougaard, J., Webb, K.J., et al. (2005). Seven Lotus japonicus genes required for transcriptional reprogramming of the root during fungal and bacterial symbiosis. Plant Cell 17, 2217-2229.

Kloepper, J.W., Ryu, C.M., and Zhang, S. (2004). Induced Systemic Resistance and Promotion of Plant Growth by Bacillus spp. Phytopathology 94, 1259-1266.

Knoester, M., Pieterse, C.M., Bol, J.F., and Van Loon, L.C. (1999). Systemic resistance in Arabidopsis induced by rhizobacteria requires ethylene-dependent signaling at the site of application. Mol Plant Microbe Interact 12, 720-727.

Kondorosi, E., Roudier, F., and Gendreau, E. (2000). Plant cell-size control: growing by ploidy? Curr Opin Plant Biol 3, 488-492.

Kosuta, S., Chabaud, M., Lougnon, G., Gough, C., Denarie, J., Barker, D.G., and Becard, G. (2003). A diffusible factor from arbuscular mycorrhizal fungi induces symbiosis-specific MtENOD11 expression in roots of *Medicago truncatula*. Plant Physiol 131, 952-962.

Kroymann, J., Textor, S., Tokuhisa, J.G., Falk, K.L., Bartram, S., Gershenzon, J., and Mitchell-Olds, T. (2001). A gene controlling variation in Arabidopsis glucosinolate composition is part of the methionine chain elongation pathway. Plant Physiol 127, 1077-1088.

Kundu, B.S., Sangwan, P., Sharma, P.K., and Nandwal, A.S. (1997). Response of pearlmillet to phytohormones produced by *Azospirillum brasilense*. Indian J Plant Physiol 2, 101-104.

Kunze, G., Zipfel, C., Robatzek, S., Niehaus, K., Boller, T., and Felix, G. (2004). The N terminus of bacterial elongation factor Tu elicits innate immunity in Arabidopsis plants. Plant Cell 16, 3496-3507.

Kutz, A., Muller, A., Hennig, P., Kaiser, W.M., Piotrowski, M., and Weiler, E.W. (2002). A role for nitrilase 3 in the regulation of root morphology in sulphur-starving *Arabidopsis thaliana*. Plant J 30, 95-106.

Lambrecht, M., Okon, Y., Vande Broek, A., and Vanderleyden, J. (2000). Indole-3-acetic acid: a reciprocal signalling molecule in bacteria-plant interactions. Trends Microbiol 8, 298-300.

Lambrecht, M., Vande Broek, A., Dosselaere, F., and Vanderleyden, J. (1999). The ipdC promoter auxin-responsive element of *Azospirillum brasilense*, a prokaryotic ancestral form of the plant AuxRE? Mol Microbiol 32, 889-891.

Lavrinenko, K., Chernousova, E., Gridneva, E., Dubinina, G., Akimov, V., Kuever, J., Lysenko, A., and Grabovich, M. (2010). *Azospirillum thiophilum* sp. nov., a novel diazotrophic bacterium isolated from a sulfide spring. Int J Syst Evol Microbiol.

Lee, C.W., Efetova, M., Engelmann, J.C., Kramell, R., Wasternack, C., Ludwig-Muller, J., Hedrich, R., and Deeken, R. (2009). *Agrobacterium tumefaciens* promotes tumor induction by modulating pathogen defense in *Arabidopsis thaliana*. Plant Cell 21, 2948-2962.

Lee, M.M., and Schiefelbein, J. (1999). WEREWOLF, a MYB-related protein in Arabidopsis, is a position-dependent regulator of epidermal cell patterning. Cell 99, 473-483.

Lee, S.H., and Cho, H.T. (2006). PINOID positively regulates auxin efflux in Arabidopsis root hair cells and tobacco cells. Plant Cell 18, 1604-1616.

Levine, A., Tenhaken, R., Dixon, R., and Lamb, C. (1994). H2O2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. Cell 79, 583-593.

LeVine, D., Kaplan, M.J., and Greenaway, P.J. (1972). The purification and characterization of wheat-germ agglutinin. Biochem J 129, 847-856.

Levy, J., Bres, C., Geurts, R., Chalhoub, B., Kulikova, O., Duc, G., Journet, E.P., Ane, J.M., Lauber, E., Bisseling, T., et al. (2004). A putative Ca2+ and calmodulin-dependent protein kinase required for bacterial and fungal symbioses. Science 303, 1361-1364.

Leyva, L.A., and Bashan, Y. (2008). Activity of two catabolic enzymes of the phosphogluconate pathway in mesquite roots inoculated with *Azospirillum brasilense* Cd. Plant Physiol Biochem 46, 898-904.

Li, J., Brader, G., and Palva, E.T. (2004). The WRKY70 transcription factor: a node of convergence for jasmonate-mediated and salicylate-mediated signals in plant defense. Plant Cell 16, 319-331.

Li, J., Ou-Lee, T.M., Raba, R., Amundson, R.G., and Last, R.L. (1993). Arabidopsis Flavonoid Mutants Are Hypersensitive to UV-B Irradiation. Plant Cell 5, 171-179.

Li, X., and Christie, P. (2001). Changes in soil solution Zn and pH and uptake of Zn by arbuscular mycorrhizal red clover in Zn-contaminated soil. Chemosphere 42, 201-207.

Lin, S.Y., Young, C.C., Hupfer, H., Siering, C., Arun, A.B., Chen, W.M., Lai, W.A., Shen, F.T., Rekha, P.D., and Yassin, A.F. (2009). *Azospirillum picis* sp. nov., isolated from discarded tar. Int J Syst Evol Microbiol 59, 761-765.

Lin, W., Okon, Y., and Hardy, R.W. (1983). Enhanced Mineral Uptake by *Zea mays* and Sorghum bicolor Roots Inoculated with *Azospirillum brasilense*. Appl Environ Microbiol 45, 1775-1779.

Lohar, D.P., Haridas, S., Gantt, J.S., and VandenBosch, K.A. (2007). A transient decrease in reactive oxygen species in roots leads to root hair deformation in the legume-rhizobia symbiosis. New Phytol 173, 39-49.

Lucangeli, C., and Bottini, R. (1997). Effects of Azospirillum spp. on endogenous gibberellin content and growth of maize (*Zea mays* L.) treated with uniconazole. Symbiosis 23, 63-72.

Lucic, E., Fourrey, C., Kohler, A., Martin, F., Chalot, M., and Brun-Jacob, A. (2008). A gene repertoire for nitrogen transporters in Laccaria bicolor. New Phytol 180, 343-364.

Lucy, M., Reed, E., and Glick, B.R. (2004). Applications of free living plant growth-promoting rhizobacteria. Antonie Van Leeuwenhoek 86, 1-25.

Madsen, E.B., Madsen, L.H., Radutoiu, S., Olbryt, M., Rakwalska, M., Szczyglowski, K., Sato, S., Kaneko, T., Tabata, S., Sandal, N., et al. (2003). A receptor kinase gene of the LysM type is involved in legume perception of rhizobial signals. Nature 425, 637-640.

Magalhles, F.M., Baldani, J.I., Souto, S.M., Kuykendall, J.R., and Dobereiner, J. (1983). A new acid-tolerant Azospirillum species. An Acad Brasil CiCnc 55, 417-430.

Mantelin, S., Desbrosses, G., Larcher, M., Tranbarger, T.J., Cleyet-Marel, J.C., and Touraine, B. (2006). Nitrate-dependent control of root architecture and N nutrition are altered by a plant growth-promoting Phyllobacterium sp. Planta 223, 591-603.

Maris, A., Suslov, D., Fry, S.C., Verbelen, J.P., and Vissenberg, K. (2009). Enzymic characterization of two recombinant xyloglucan endotransglucosylase/hydrolase (XTH) proteins of Arabidopsis and their effect on root growth and cell wall extension. J Exp Bot 60, 3959-3972.

Marks, M.D., Wenger, J.P., Gilding, E., Jilk, R., and Dixon, R.A. (2009). Transcriptome analysis of Arabidopsis wild-type and gl3-sst sim trichomes identifies four additional genes required for trichome development. Mol Plant 2, 803-822.

Martin, G.B., Bogdanove, A.J., and Sessa, G. (2003). Understanding the functions of plant disease resistance proteins. Annu Rev Plant Biol 54, 23-61.

Mathesius, U., Schlaman, H.R., Spaink, H.P., Of Sautter, C., Rolfe, B.G., and Djordjevic, M.A. (1998). Auxin transport inhibition precedes root nodule formation in white clover roots and is regulated by flavonoids and derivatives of chitin oligosaccharides. Plant J 14, 23-34.

Mathis, R., Van Gijsegem, F., De Rycke, R., D'Haeze, W., Van Maelsaeke, E., Anthonio, E., Van Montagu, M., Holsters, M., and Vereecke, D. (2005). Lipopolysaccharides as a communication signal for progression of legume endosymbiosis. Proc Natl Acad Sci U S A 102, 2655-2660.

Matsui, K., Umemura, Y., and Ohme-Takagi, M. (2008). AtMYBL2, a protein with a single MYB domain, acts as a negative regulator of anthocyanin biosynthesis in Arabidopsis. Plant J 55, 954-967.

Mehnaz, S., Weselowski, B., and Lazarovits, G. (2007a). *Azospirillum canadense* sp. nov., a nitrogen-fixing bacterium isolated from corn rhizosphere. Int J Syst Evol Microbiol 57, 620-624.

Mehnaz, S., Weselowski, B., and Lazarovits, G. (2007b). *Azospirillum zeae* sp. nov., a diazotrophic bacterium isolated from rhizosphere soil of *Zea mays*. Int J Syst Evol Microbiol 57, 2805-2809.

Menand, B., Yi, K., Jouannic, S., Hoffmann, L., Ryan, E., Linstead, P., Schaefer, D.G., and Dolan, L. (2007). An ancient mechanism controls the development of cells with a rooting function in land plants. Science 316, 1477-1480.

Mirouze, M., Sels, J., Richard, O., Czernic, P., Loubet, S., Jacquier, A., Francois, I.E., Cammue, B.P., Lebrun, M., Berthomieu, P., et al. (2006). A putative novel role for plant defensins: a defensin from the zinc hyper-accumulating plant, *Arabidopsis halleri*, confers zinc tolerance. Plant J 47, 329-342.

Molina-Favero, C., Creus, C.M., Simontacchi, M., Puntarulo, S., and Lamattina, L. (2008). Aerobic nitric oxide production by *Azospirillum brasilense* Sp245 and its influence on root architecture in tomato. Mol Plant Microbe Interact 21, 1001-1009.

Molla, A.H., Shamsuddin, Z.H., and Saud, H.M. (2001). Mechanism of root growth and promotion of nodulation in vegetable soybean by *Azospirillum brasilense*. Commun Soil Sci Plant Anal 32, 2177–2187.

Mowla, S.B., Cuypers, A., Driscoll, S.P., Kiddle, G., Thomson, J., Foyer, C.H., and Theodoulou, F.L. (2006). Yeast complementation reveals a role for an *Arabidopsis thaliana* late embryogenesis abundant (LEA)-like protein in oxidative stress tolerance. Plant J 48, 743-756.

Mravec, J., Skupa, P., Bailly, A., Hoyerova, K., Krecek, P., Bielach, A., Petrasek, J., Zhang, J., Gaykova, V., Stierhof, Y.D., et al. (2009). Subcellular homeostasis of phytohormone auxin is mediated by the ER-localized PIN5 transporter. Nature 459, 1136-1140.

Muller, A., Guan, C., Galweiler, L., Tanzler, P., Huijser, P., Marchant, A., Parry, G., Bennett, M., Wisman, E., and Palme, K. (1998). AtPIN2 defines a locus of Arabidopsis for root gravitropism control. EMBO J 17, 6903-6911.

Murray, S.L., Ingle, R.A., Petersen, L.N., and Denby, K.J. (2007). Basal resistance against *Pseudomonas syringae* in Arabidopsis involves WRKY53 and a protein with homology to a nematode resistance protein. Mol Plant Microbe Interact 20, 1431-1438.

Nakajima, J., Sato, Y., Hoshino, T., Yamazaki, M., and Saito, K. (2006). Mechanistic study on the oxidation of anthocyanidin synthase by quantum mechanical calculation. J Biol Chem 281, 21387-21398.

Nehl, D.B., Allen, S.J., and Brown, J.F. (1996). Deleterious rhizosphere bacteria: an integrating perspective. Appl Soil Ecol 5, 1-20.

Nehls, U., Wiese, J., Guttenberger, M., and Hampp, R. (1998). Carbon allocation in ectomycorrhizas: identification and expression analysis of an Amanita muscaria monosaccharide transporter. Mol Plant Microbe Interact 11, 167-176.

Noel, L.D., Cagna, G., Stuttmann, J., Wirthmuller, L., Betsuyaku, S., Witte, C.P., Bhat, R., Pochon, N., Colby, T., and Parker, J.E. (2007). Interaction between SGT1 and cytosolic/nuclear HSC70 chaperones regulates Arabidopsis immune responses. Plant Cell 19, 4061-4076.

Norholm, M.H., Nour-Eldin, H.H., Brodersen, P., Mundy, J., and Halkier, B.A. (2006). Expression of the Arabidopsis high-affinity hexose transporter STP13 correlates with programmed cell death. FEBS Lett 580, 2381-2387.

Nurnberger, T., Brunner, F., Kemmerling, B., and Piater, L. (2004). Innate immunity in plants and animals: striking similarities and obvious differences. Immunol Rev 198, 249-266.

Okamoto, M., Kumar, A., Li, W., Wang, Y., Siddiqi, M.Y., Crawford, N.M., and Glass, A.D. (2006). High-affinity nitrate transport in roots of Arabidopsis depends on expression of the NAR2-like gene AtNRT3.1. Plant Physiol 140, 1036-1046.

Oldroyd, G.E., and Downie, J.A. (2004). Calcium, kinases and nodulation signalling in legumes. Nat Rev Mol Cell Biol 5, 566-576.

Oldroyd, G.E., Harrison, M.J., and Udvardi, M. (2005). Peace talks and trade deals. Keys to long-term harmony in legume-microbe symbioses. Plant Physiol 137, 1205-1210.

Orozco-Cardenas, M.L., Narvaez-Vasquez, J., and Ryan, C.A. (2001). Hydrogen peroxide acts as a second messenger for the induction of defense genes in tomato plants in response to wounding, systemin, and methyl jasmonate. Plant Cell 13, 179-191.

Osato, Y., Yokoyama, R., and Nishitani, K. (2006). A principal role for AtXTH18 in *Arabidopsis thaliana* root growth: a functional analysis using RNAi plants. J Plant Res 119, 153-162.

Patriquin, D.G., and Dobereiner, J. (1978). Light microscopy observations of tetrazolium-reducing bacteria in the endorhizosphere of maize and other grasses in Brazil. Can J Microbiol 24, 734-742.

Patriquin, D.G., Döbereiner, J., and Jain, D.K. (1983). Sites and processes of association between diazotrophs and grasses. Can J Microbiol 29, 900-915.

Peng, G., Wang, H., Zhang, G., Hou, W., Liu, Y., Wang, E.T., and Tan, Z. (2006). *Azospirillum melinis* sp. nov., a group of diazotrophs isolated from tropical molasses grass. Int J Syst Evol Microbiol 56, 1263-1271.

Pereg Gerk, L., Gilchrist, K., and Kennedy, I.R. (2000). Mutants with enhanced nitrogenase activity in hydroponic *Azospirillum brasilense*-wheat associations. Appl Environ Microbiol 66, 2175-2184.

Peret, B., Swarup, R., Jansen, L., Devos, G., Auguy, F., Collin, M., Santi, C., Hocher, V., Franche, C., Bogusz, D., et al. (2007). Auxin influx activity is associated with Frankia infection during actinorhizal nodule formation in Casuarina glauca. Plant Physiol 144, 1852-1862.

Pereyra, M.A., Zalazar, C.A., and Barassi, C.A. (2006). Root phospholipids in Azospirillum-inoculated wheat seedlings exposed to water stress. Plant Physiol Biochem 44, 873-879.

Perrig, D., Boiero, M.L., Masciarelli, O.A., Penna, C., Ruiz, O.A., Cassan, F.D., and Luna, M.V. (2007). Plant-growth-promoting compounds produced by two agronomically important strains of *Azospirillum brasilense*, and implications for inoculant formulation. Appl Microbiol Biotechnol 75, 1143-1150.

Pfalz, M., Vogel, H., and Kroymann, J. (2009). The gene controlling the indole glucosinolate modifier1 quantitative trait locus alters indole glucosinolate structures and aphid resistance in Arabidopsis. Plant Cell 21, 985-999.

Pieterse, C.M., van Wees, S.C., Hoffland, E., van Pelt, J.A., and van Loon, L.C. (1996). Systemic resistance in Arabidopsis induced by biocontrol bacteria is independent of salicylic acid accumulation and pathogenesis-related gene expression. Plant Cell 8, 1225-1237.

Pilet, P.E., and Saugy, M. (1985). Effects of applied and endogenous IAA on maize root growth. Planta, 164, 254-258.

Piotrowski, M., Schemenewitz, A., Lopukhina, A., Muller, A., Janowitz, T., Weiler, E.W., and Oecking, C. (2004). Desulfoglucosinolate sulfotransferases from *Arabidopsis thaliana* catalyze the final step in the biosynthesis of the glucosinolate core structure. J Biol Chem 279, 50717-50725.

Pogany, M., von Rad, U., Grun, S., Dongo, A., Pintye, A., Simoneau, P., Bahnweg, G., Kiss, L., Barna, B., and Durner, J. (2009). Dual roles of reactive oxygen species and NADPH oxidase RBOHD in an Arabidopsis-Alternaria pathosystem. Plant Physiol 151, 1459-1475.

Pollmann, S., Neu, D., and Weiler, E.W. (2003). Molecular cloning and characterization of an amidase from *Arabidopsis thaliana* capable of converting indole-3-acetamide into the plant growth hormone, indole-3-acetic acid. Phytochemistry 62, 293-300.

Pozo, M.J., Van Der Ent, S., Van Loon, L.C., and Pieterse, C.M. (2008). Transcription factor MYC2 is involved in priming for enhanced defense during rhizobacteria-induced systemic resistance in *Arabidopsis thaliana*. New Phytol 180, 511-523.

Prabhakar, V., Lottgert, T., Gigolashvili, T., Bell, K., Flugge, U.I., and Hausler, R.E. (2009). Molecular and functional characterization of the plastid-localized Phosphoenolpyruvate enolase (ENO1) from *Arabidopsis thaliana*. FEBS Lett 583, 983-991.

Prell, J., and Poole, P. (2006). Metabolic changes of rhizobia in legume nodules. Trends Microbiol 14, 161-168.

Prinsen, E., Chauvaux, N., Schmidt, J., John, M., Wieneke, U., De Greef, J., Schell, J., and Van Onckelen, H. (1991). Stimulation of indole-3-acetic acid production in Rhizobium by flavonoids. FEBS Lett 282, 53-55.

Prinsen, E., Costacurta, A., Michiels, K., Vanderleyden, J., and Van Onckelen, H. (1993). *Azospirillum brasilense* indole-3-acetic acid biosynthesis:evidence for a non-tryptophan dependent pathway. Plant-Microbe Interact 6, 609-615.

Ramu, S.K., Peng, H.M., and Cook, D.R. (2002). Nod factor induction of reactive oxygen species production is correlated with expression of the early nodulin gene rip1 in *Medicago truncatula*. Mol Plant Microbe In 15, 522-528.

Reinhold, B., Hurek, T., Fendrik, I., Pot, B., Gillis, M., Kersters, K., Thielemans, S., and Deley, J. (1987). *Azospirillum halopraeferens* sp. nov, a nitrogen fixing organism associated with roots of kallar grass (*Leptochloa fusca* (L) Kunth). Int J Syst Bacteriol 37, 43-51.

Rodriguez-Navarro, D.N., Dardanelli, M.S., and Ruiz-Sainz, J.E. (2007). Attachment of bacteria to the roots of higher plants. FEMS Microbiol Lett 272, 127-136.

Rubio, M.C., James, E.K., Clemente, M.R., Bucciarelli, B., Fedorova, M., Vance, C.P., and Becana, M. (2004). Localization of superoxide dismutases and hydrogen peroxide in legume root nodules. Mol Plant Microbe Interact 17, 1294-1305.

Rudrappa, T., Czymmek, K.J., Pare, P.W., and Bais, H.P. (2008). Root-secreted malic acid recruits beneficial soil bacteria. Plant Physiol 148, 1547-1556.

Rudrappa, T., Quinn, W.J., Stanley-Wall, N.R., and Bais, H.P. (2007). A degradation product of the salicylic acid pathway triggers oxidative stress resulting in down-regulation of *Bacillus subtilis* biofilm formation on *Arabidopsis thaliana* roots. Planta 226, 283-297.

Ryu, C.M., Murphy, J.F., Mysore, K.S., and Kloepper, J.W. (2004). Plant growth-promoting rhizobacteria systemically protect *Arabidopsis thaliana* against Cucumber mosaic virus by a salicylic acid and NPR1-independent and jasmonic acid-dependent signaling pathway. Plant J 39, 381-392.

Sanguin, H., Sarniguet, A., Gazengel, K., Moenne-Loccoz, Y., and Grundmann, G.L. (2009). Rhizosphere bacterial communities associated with disease suppressiveness stages of take-all decline in wheat monoculture. New Phytol 184, 694-707.

Santos, R., Herouart, D., Sigaud, S., Touati, D., and Puppo, A. (2001). Oxidative burst in alfalfa-*Sinorhizobium meliloti* symbiotic interaction. Mol Plant Microbe In 14, 86-89.

Saxena, B., Modi, M., and Modi, V.V. (1986). Isolation and characterization of siderophore from *Azospirillum lipoferum* D2. J Gen Microbiol 132, 2219-2224.

Schauser, L., Roussis, A., Stiller, J., and Stougaard, J. (1999). A plant regulator controlling development of symbiotic root nodules. Nature 402, 191-195.

Scheres, B., Van De Wiel, C., Zalensky, A., Horvath, B., Spaink, H., Van Eck, H., Zwartkruis, F., Wolters, A.M., Gloudemans, T., Van Kammen, A., et al. (1990). The ENOD12 gene product is involved in the infection process during the pea-Rhizobium interaction. Cell 60, 281-294.

Schloter, M., and Hartmann, A. (1998). Endophytic and surface colonization of wheat roots (Triticum aestivum) by different *Azospirillum brasilense* strains studied with strain-specific monoclonal antibodies. Symbiosis 25.

Schultz, C.J., Rumsewicz, M.P., Johnson, K.L., Jones, B.J., Gaspar, Y.M., and Bacic, A. (2002). Using genomic resources to guide research directions. The arabinogalactan protein gene family as a test case. Plant Physiol 129, 1448-1463.

Schussler, A., Martin, H., Cohen, D., Fitz, M., and Wipf, D. (2006). Characterization of a carbohydrate transporter from symbiotic glomeromycotan fungi. Nature 444, 933-936.

Schuster, J., Knill, T., Reichelt, M., Gershenzon, J., and Binder, S. (2006). Branched-chain aminotransferase4 is part of the chain elongation pathway in the biosynthesis of methionine-derived glucosinolates in Arabidopsis. Plant Cell 18, 2664-2679.

Segarra, G., Van der Ent, S., Trillas, I., and Pieterse, C.M. (2009). MYB72, a node of convergence in induced systemic resistance triggered by a fungal and a bacterial beneficial microbe. Plant Biol (Stuttg) 11, 90-96.

Selosse, M.A., and Roy, M. (2009). Green plants that feed on fungi: facts and questions about mixotrophy. Trends Plant Sci 14, 64-70.

Sessitsch, A., Howieson, J.G., Perret, X., Antoun, H., and Martinez-Romero, E. (2002). Advances in Rhizobium research. Crit Rev Plant Sci 21, 323-378.

Shoresh, M., Yedidia, I., and Chet, I. (2005). Involvement of Jasmonic Acid/Ethylene Signaling Pathway in the Systemic Resistance Induced in Cucumber by Trichoderma asperellum T203. Phytopathology 95, 76-84.

Singh, B.K., Millard, P., Whiteley, A.S., and Murrell, J.C. (2004). Unravelling rhizosphere-microbial interactions: opportunities and limitations. Trends Microbiol 12, 386-393.

Sly, L., and Stackebrandt, E. (1999). Description of Skermanella parooensis gen. nov., sp. nov. to accommodate *Conglomeromonas largomobilis* subsp. parooensis following the transfer of *Conglomeromonas largomobilis* subsp. largomobilis to the genus Azospirillum. Int J Syst Bacteriol 49, 541-544.

Smith, S., and Read, D. (2008). mycorrhizal symbiosis. New York Academic 3rd ed.

Somers, E., D. Ptacek, P. Gysegom, M. Srinivasan, and Vanderleyden, J. (2005). *Azospirillum brasilense* produces the auxin-like phenylacetic acid by using the key enzyme for indole-3-acetic acid biosynthesis. Appl EnvironMicrobiol Res 71, 1803–1810.

Somers, E., Vanderleyden, J., and Srinivasan, M. (2004). Rhizosphere bacterial signalling: a love parade beneath our feet. Crit Rev Microbiol 30, pp. 205-240.

Soto, M.J., Dominguez-Ferreras, A., Perez-Mendoza, D., Sanjuan, J., and Olivares, J. (2009). Mutualism versus pathogenesis: the give-and-take in plant-bacteria interactions. Cell Microbiol 11, 381-388.

Sprent, J.I. (2007). Evolving ideas of legume evolution and diversity: a taxonomic perspective on the occurrence of nodulation. New Phytol 174, 11-25.

Steenhoudt, O., and Vanderleyden, J. (2000). Azospirillum, a free-living nitrogen-fixing bacterium closely associated with grasses: genetic, biochemical and ecological aspects. FEMS Microbiol Rev 24, 487-506.

Sticher, L., Mauch-Mani, B., and Metraux, J.P. (1997). Systemic acquired resistance. Annu Rev Phytopathol 35, 235-270.

Takeda, S., Gapper, C., Kaya, H., Bell, E., Kuchitsu, K., and Dolan, L. (2008). Local positive feedback regulation determines cell shape in root hair cells. Science 319, 1241-1244.

Talalay, P., and Fahey, J.W. (2001). Phytochemicals from cruciferous plants protect against cancer by modulating carcinogen metabolism. J Nutr 131, 3027S-3033S.

Tarrand, J., Krieg, N., and J.Döbereiner (1978). A taxonomic study of the Spirillum lipoferum group, with descriptions of a new genus, Azospirillum gen. nov. and two species, *Azospirillum lipoferum* (Beijerinck) comb. nov. and *Azospirillum brasilense* sp. nov.

. Can J Microbiol 8, 967-980.

Terras, F.R., Eggermont, K., Kovaleva, V., Raikhel, N.V., Osborn, R.W., Kester, A., Rees, S.B., Torrekens, S., Van Leuven, F., Vanderleyden, J., et al. (1995). Small cysteine-rich antifungal proteins from radish: their role in host defense. Plant Cell 7, 573-588.

Textor, S., Bartram, S., Kroymann, J., Falk, K.L., Hick, A., Pickett, J.A., and Gershenzon, J. (2004). Biosynthesis of methionine-derived glucosinolates in *Arabidopsis thaliana*: recombinant expression and characterization of methylthioalkylmalate synthase, the condensing enzyme of the chain-elongation cycle. Planta 218, 1026-1035.

Textor, S., de Kraker, J.W., Hause, B., Gershenzon, J., and Tokuhisa, J.G. (2007). MAM3 catalyzes the formation of all aliphatic glucosinolate chain lengths in Arabidopsis. Plant Physiol 144, 60-71.

Thimm, O., Blasing, O., Gibon, Y., Nagel, A., Meyer, S., Kruger, P., Selbig, J., Muller, L.A., Rhee, S.Y., and Stitt, M. (2004). MAPMAN: a user-driven tool to display genomics data sets onto diagrams of metabolic pathways and other biological processes. Plant J 37, 914-939.

Thomma, B.P., Cammue, B.P., and Thevissen, K. (2002). Plant defensins. Planta 216, 193-202.

Tien, T.M., Gaskins, M.H., and Hubbell, D.H. (1979). Plant Growth Substances Produced by *Azospirillum brasilense* and Their Effect on the Growth of Pearl Millet (Pennisetum americanum L.). Appl Environ Microbiol 37, 1016-1024.

Tierens, K.F., Thomma, B.P., Brouwer, M., Schmidt, J., Kistner, K., Porzel, A., Mauch-Mani, B., Cammue, B.P., and Broekaert, W.F. (2001). Study of the role of antimicrobial glucosinolate-derived isothiocyanates in resistance of Arabidopsis to microbial pathogens. Plant Physiol 125, 1688-1699.

Tjamos, S.E., Flemetakis, E., Paplomatas, E.J., and Katinakis, P. (2005). Induction of resistance to Verticillium dahliae in *Arabidopsis thaliana* by the biocontrol agent K-165 and pathogenesis-related proteins gene expression. Mol Plant Microbe Interact 18, 555-561.

Tohge, T., Nishiyama, Y., Hirai, M.Y., Yano, M., Nakajima, J., Awazuhara, M., Inoue, E., Takahashi, H., Goodenowe, D.B., Kitayama, M., et al. (2005). Functional genomics by integrated analysis of metabolome and transcriptome of Arabidopsis plants over-expressing an MYB transcription factor. Plant J 42, 218-235.

Torres, M.A., Dangl, J.L., and Jones, J.D. (2002). Arabidopsis gp91phox homologues AtrbohD and AtrbohF are required for accumulation of reactive oxygen intermediates in the plant defense response. Proc Natl Acad Sci U S A 99, 517-522.

Umali-Garcia, M., Hubbell, D.H., Gaskins, M.H., and Dazzo, F.B. (1980). Association of Azospirillum with Grass Roots. Appl Environ Microbiol 39, 219-226.

Van Bastelaere, E., Lambrecht, M., Vermeiren, H., Van Dommelen, A., Keijers, V., Proost, P., and Vanderleyden, J. (1999). Characterization of a sugar-binding protein from *Azospirillum brasilense* by using Tn5-lacZ. Appl Environ Microbiol 56, 990-996.

Van de Broek, Michiels, A., J., Van Gool, A., and J., V. (1993). Spatial-temporal colonization patterns of *Azospirillum brasilense* on the wheat root surface and expression of the bacterial nifH-gene during association. Molec Plant-Microbe Interact 6, 592–600.

Van der Ent, S., Verhagen, B.W., Van Doorn, R., Bakker, D., Verlaan, M.G., Pel, M.J., Joosten, R.G., Proveniers, M.C., Van Loon, L.C., Ton, J., et al. (2008). MYB72 is required in early signaling steps of rhizobacteria-induced systemic resistance in Arabidopsis. Plant Physiol 146, 1293-1304.

Van Dommelen, A., Keijers, V., Vanderleyden, J., and de Zamaroczy, M. (1998). (Methyl)ammonium transport in the nitrogen-fixing bacterium *Azospirillum brasilense*. J Bacteriol 180, 2652-2659.

van Loon, L.C., Bakker, P.A., and Pieterse, C.M. (1998). Systemic resistance induced by rhizosphere bacteria. Annu Rev Phytopathol 36, 453-483.

van Loon, L.C., Bakker, P.A., van der Heijdt, W.H., Wendehenne, D., and Pugin, A. (2008). Early responses of tobacco suspension cells to rhizobacterial elicitors of induced systemic resistance. Mol Plant Microbe Interact 21, 1609-1621.

van Rhijn, P., Fang, Y., Galili, S., Shaul, O., Atzmon, N., Wininger, S., Eshed, Y., Lum, M., Li, Y., To, V., et al. (1997). Expression of early nodulin genes in alfalfa mycorrhizae indicates that signal transduction pathways used in forming arbuscular mycorrhizae and Rhizobium-induced nodules may be conserved. Proc Natl Acad Sci U S A 94, 5467-5472.

Vande Broek, A., Lambrecht, M., and Vanderleyden, J. (1998). Bacterial chemotactic motility is important for the initiation of wheat root colonization by *Azospirillum brasilense*. Microbiology 144 (Pt 9), 2599-2606.

Vercammen, J., Pham-Tuan, H., Arickx, I., Van der Straeten, D., and Sandra, P. (2001). Monitoring of isothiocyanates emanating from *Arabidopsis thaliana* upon paraquat spraying. J Chromatogr A 912, 127-134.

Von Bulow, J.F., and Dobereiner, J. (1975). Potential for Nitrogen Fixation in Maize Genotypes in Brazil. Proc Natl Acad Sci U S A 72, 2389-2393.

Vorwerk, S., Biernacki, S., Hillebrand, H., Janzik, I., Muller, A., Weiler, E.W., and Piotrowski, M. (2001). Enzymatic characterization of the recombinant *Arabidopsis thaliana* nitrilase subfamily encoded by the NIT2/NIT1/NIT3-gene cluster. Planta 212, 508-516.

Vorwieger, A., Gryczka, C., Czihal, A., Douchkov, D., Tiedemann, J., Mock, H.P., Jakoby, M., Weisshaar, B., Saalbach, I., and Baumlein, H. (2007). Iron assimilation and transcription factor controlled synthesis of riboflavin in plants. Planta 226, 147-158.

Wada, T., Tachibana, T., Shimura, Y., and Okada, K. (1997). Epidermal cell differentiation in Arabidopsis determined by a Myb homolog, CPC. Science 277, 1113-1116.

Wang, H.Y., Klatte, M., Jakoby, M., Baumlein, H., Weisshaar, B., and Bauer, P. (2007). Iron deficiency-mediated stress regulation of four subgroup Ib BHLH genes in *Arabidopsis thaliana*. Planta 226, 897-908.

Wasson, A.P., Pellerone, F.I., and Mathesius, U. (2006). Silencing the flavonoid pathway in *Medicago truncatula* inhibits root nodule formation and prevents auxin transport regulation by rhizobia. Plant Cell 18, 1617-1629.

Webster, G., Jain, V., Davey, M.R., Gough, C., Vasse, J., Dénarié, J., and Cocking, E.C. (1998). The flavonoid naringenin stimulates the intercellular colonization of wheat roots by *Azorhizobium caulinodans*. Plant, Cell & Environment 21, 373-383(311).

Westby, C.A., Enderlin, C.S., Steinberg, N.A., Joseph, C.M., and Meeks, J.C. (1987). Assimilation of 13NH4+ by *Azospirillum brasilense* grown under nitrogen limitation and excess. J Bacteriol 169, 4211-4214.

Woodward, A.W., and Bartel, B. (2005). Auxin: regulation, action, and interaction. Ann Bot 95, 707-735.

Xie, C.H., and Yokota, A. (2005). *Azospirillum oryzae* sp. nov., a nitrogen-fixing bacterium isolated from the roots of the rice plant *Oryza sativa*. Int J Syst Evol Microbiol 55, 1435-1438.

Yan, Z., Reddy, M.S., Ryu, C.M., McInroy, J.A., Wilson, M., and Kloepper, J.W. (2002). Induced systemic protection against tomato late blight elicited by plant growth-promoting rhizobacteria. Phytopathology 92, 1329-1333.

Yanhui, C., Xiaoyuan, Y., Kun, H., Meihua, L., Jigang, L., Zhaofeng, G., Zhiqiang, L., Yunfei, Z., Xiaoxiao, W., Xiaoming, Q., et al. (2006). The MYB transcription factor superfamily of Arabidopsis: expression analysis and phylogenetic comparison with the rice MYB family. Plant Mol Biol 60, 107-124.

Yasuda, M., Isawa, T., Shinozaki, S., Minamisawa, K., and Nakashita, H. (2009). Effects of colonization of a bacterial endophyte, *Azospirillum sp.* B510, on disease resistance in rice. Biosci Biotechnol Biochem 73, 2595-2599.

Yegorenkova, I.V., Konnova, S.A., Sachuk, V.N., Ignatov, V.V., and . (2001). *Azospirillum brasilense* colonization of wheat roots and the role of lectin-carbohydrate interactions in bacterial adsorption and root-hair deformation. Plant Soil 231, 275–282.

Yi, K., Menand, B., Bell, E., and Dolan, L. (2010). A basic helix-loop-helix transcription factor controls cell growth and size in root hairs. Nat Genet 42, 264-267.

Yonekura-Sakakibara, K., Tohge, T., Niida, R., and Saito, K. (2007). Identification of a flavonol 7-O-rhamnosyltransferase gene determining flavonoid pattern in Arabidopsis by transcriptome coexpression analysis and reverse genetics. J Biol Chem 282, 14932-14941.

Young, C.C., Hupfer, H., Siering, C., Ho, M.J., Arun, A.B., Lai, W.A., Rekha, P.D., Shen, F.T., Hung, M.H., Chen, W.M., et al. (2008). *Azospirillum rugosum* sp. nov., isolated from oil-contaminated soil. Int J Syst Evol Microbiol 58, 959-963.

Zhang, H., Jennings, A., Barlow, P.W., and Forde, B.G. (1999). Dual pathways for regulation of root branching by nitrate. Proc Natl Acad Sci U S A 96, 6529-6534.

Zhang, J., Subramanian, S., Stacey, G., and Yu, O. (2009). Flavones and flavonols play distinct critical roles during nodulation of *Medicago truncatula* by *Sinorhizobium meliloti*. Plant J 57, 171-183.

Zhang, Z., Ober, J.A., and Kliebenstein, D.J. (2006). The gene controlling the quantitative trait locus EPITHIOSPECIFIER MODIFIER1 alters glucosinolate hydrolysis and insect resistance in Arabidopsis. Plant Cell 18, 1524-1536.

Zhou, Y., Wei, W., Wang, X., Xu, L., and Lai, R. (2009). *Azospirillum palatum* sp. nov., isolated from forest soil in Zhejiang province, China. J Gen Appl Microbiol 55, 1-7.

Zhu, Y., Christie, P., and Laidlaw, A.S. (2001). Uptake of Zn by arbuscular mycorrhizal white clover from Zn-contaminated soil. Chemosphere 42, 193-199.

Zimmermann, P., Hirsch-Hoffmann, M., Hennig, L., and Gruissem, W. (2004). GENEVESTIGATOR. Arabidopsis microarray database and analysis toolbox. Plant Physiol 136, 2621-2632.

Zipfel, C., and Felix, G. (2005). Plants and animals: a different taste for microbes? Curr Opin Plant Biol 8, 353-360.

Zipfel, C., Kunze, G., Chinchilla, D., Caniard, A., Jones, J.D., Boller, T., and Felix, G. (2006). Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts Agrobacterium-mediated transformation. Cell 125, 749-760.

6 Supplement

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Physiological and molecular basis of *Azospirillum-Arabidopsis* Interaction

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Hiermit erkläre ich, dass ich, Nazeer Ahmed, die vorliegende Dissertation in allen Teilen selbständig und ausschließlich mit den angegebenen Hilfsmitteln angefertigt habe. Alle Stellen, die gemäß Wortlaut oder Inhalt aus anderen Arbeiten entnommen sind, wurden durch Angabe der Quelle kenntlich gemacht.

Ich habe die Dissertation weder in gleicher noch in ähnlicher Form in anderen Prüfungsverfahren vorgelegt. Außerdem habe ich bislang noch keine weiteren akademischen Grade erworben oder zu erwerben versucht.

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