The Molecular Analysis of the Effects of Lumichrome as a Plant Growth Promoting Substance

by

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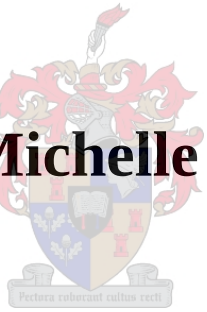

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Declaration

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Signature: 

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Summary

Through powerful signal molecules, rhizobacteria affect fundamental processes in plants. In recent years, a number of novel rhizobial molecules have been identified that positively affect plant growth and development. Previous studies have shown that *Sinorhizobium meliloti,* which form symbiotic relationships with leguminous plants, increases CO_2 availability by enhancing root respiration in alfalfa. The active compound was identified as lumichrome, a previously unrecognized rhizosphere signal molecule that has been shown to promote plant growth in various studies. Lumichrome is a common breakdown product of riboflavin and produced by both chemical and biological factors. Various studies on lumichrome have proven its growth promoting effect in the interaction with plants. The mechanism through which lumichrome increases plant growth remains to be clarified.

This study provides new insight into the molecular effects of the plant growth promoter lumichrome on the root metabolism of plants. The main aim of the work presented in this thesis was to investigate the molecular mechanism of the plant growth promoting substance lumichrome in the roots of the model plants *Lotus japonicus* and *Solanum lycopersicon* (tomato). To asses the impact of lumichrome on the root metabolism of *Lotus japonicus* and tomato and identify key genes involved in the growth stimulation, a comprehensive profile of differentially expressed genes, proteins and metabolites was compiled. As the effects of lumichrome as a plant growth promoter have not previously been tested on *Lotus japonicus* and tomato, basic growth studies were completed to determine if lumichrome indeed elicits plant growth at nanomolar concentrations, as was proven in numerous previous studies. Both *Lotus japonicus* and tomato showed significant increases in root biomass when treated with 5 nM of lumichrome. The treatment with lumichrome caused complex changes in gene expression. Generally, transcript profiling showed that the categories that were predominantly affected by lumichrome in both *Lotus* and tomato, were genes associated with RNA regulation of transcription and signaling, protein synthesis/degradation/modification and stress and defence. Proteomic studies revealed that the majority of the differentially expressed proteins were down-regulated. Lumichrome seems to largely influence proteins involved in protein folding and down-regulate proteins involved in glycolysis. Proteomics studies revealed that GS1 (*Lotus*) and GAPDH (*Lotus* and tomato) were present in lower abundance in lumichrome treated roots, therefore targeted analysis utilizing northern blots, western blots

and the measurement of enzyme activities were completed to determine and verify their specific role in the lumichrome mediated growth promotion. The results indicated that GAPDH and GS1 seem to be under post-translational modification. The influence of lumichrome on the metabolome of *Lotus* roots was immense, however minute in tomato roots.

The knowledge gained in the parallel analyses of both *Lotus japonicus* and tomato aided us in finding key genes involved in the growth stimulation. Overall, one of the most significant observations was that for the first time to our knowledge, six genes related to defence and pathogen responses were identified that are concurrently expressed in both *Lotus* and tomato. Through identifying a small number of genes involved in mediating the growth stimulation, these can be used for their functional analysis in the future, using reverse genetics to provide more insight into the molecular mechanisms that are triggered by lumichrome as a plant growth promoter.

Opsomming

Deur kragtige sein-molekules, beïnvloed rhizobakterieë basiese prosesse in plante. In die laaste jare is ’n aantal nuwe molekules, afkomstig van rhizobakterieë, geidentifiseer wat plantgroei en ontwikkeling positief beïnvloed. Voorafgaande studies het bewys dat *Sinorhizobium meliloti*, wat simbiotiese verhoudings met peulplante aangaan, die beskikbaarheid van CO_2 vermeerder deur wortel respirasie in alfalfa te verhoog. Die aktiewe komponent is as lumikroom geidentifiseer, ‘n vroeë onerkenbare risosfeer sein-molekule, wat deur vorige studies bewys is dat dit plantgroei stimuleer. Lumikroom is ’n algemene afbreekproduk van riboflavin en word geproduseer deur chemiese en biologiese faktore. Verskeie studies op lumikroom het bewys dat dit ‘n groei stimuleerende effek het op die groei van plante as dit daarmee in wisselwerking tree. Die meganisme waarmee lumikroom plante groei verhoog, is nog nie opgeklaar nie.

Hierdie studie verleen nuwe insigte in die molekulêre effekte van die plantgroei stimuleerende molekuul lumikroom op die wortel metabolisme van plante. Die hoofdoel van die werk wat voorgestel word in hierdie tesis, was om die molekulêre meganisme van die plantgroei stimuleerende stof, genaamd lumikroom, in die wortels van die model plante *Lotus japonicus* en *Solanum lycopersicon* (tamatie), te ondersoek. Om die uitwerking van lumikroom op die wortel metabolisme van *Lotus japonicus* en tamatie te bepaal, asook sleutelgene wat betrokke is by die groei stimulasie te identifiseer, is ‘n breedvoerige profiel van differensiële uitgedrukte gene, proteïne en metaboliete saamgestel. Die effekte van lumikroom as ‘n plantgroei stimuleerende stof is nog nooit op *Lotus japonicus* en tamatie getoets nie. Om díe rede is eers basiese plantgroei studies gedoen, om vas te stel of lumikroom inderdaad plantgroei teen nanomolare konsentrasies stimuleer, soos in vele voorafgaande studies bevestig is. Beide *Lotus japonicus* en tamatie het aansienlike verhogings in wortel biomassa getoon as dit met 5 nM lumikroom behandel is.

Die behandeling van plante met lumikroom het komplekse veranderinge in geen-uitdrukking veroorsaak. Oor die algemeen het die transkrip-profiele gewys dat die kategorieë wat die meeste geraak is deur lumikroom behandeling, in beide *Lotus* en tamatie, gene was wat geassosieer word met RNS regulasie van transkripsie en sein-netwerke, proteïen sintese/degradasie/wysiging en stres en verdedigings prosesse in plante. Proteïen studies het

gewys dat daar ‘n daling in die meerderheid van die proteïen vlakke was wat differensieël uitgedruk was. Dit blyk dat lumikroom in ‘n groot mate proteïene beïnvloed wat betrokke is by proteïen-vouing en veroorsaak dat proteïen vlakke van glikolitiese ensieme daal. Proteïen studies het gewys dat GS1 en GAPDH in laer vlakke teenwoordig was in lumikroom behandelde plante en daarom is ‘n meer doelgerigte analiese gedoen deur gebruik te maak van “northern blot”, “western blot” en deur die ensiem aktiwiteite te meet om hulle spesifieke rol in die lumikroom bemiddelde groei vas te stel. Die resultate wys daarop dat GAPDH en GS1 mag onder die invloed van na-translasionele verandering wees. Die invloed van lumikroom op die metabolietvlakke was groot in *Lotus* wortels, maar dit het minder van ‘n effek gehad op tamatie wortels.

Die kennis wat opgedoen is deur die paralelle analiese van beide *Lotus japonicus* en tamatie plante help ons om sleutel gene wat betrokke is by groeistimulasie te identifiseer. Een van die betekenisvolste waarnemings van hierdie studie was dat vir die eerste keer, sover ons kennis strek, ses gene wat almal betrekking het tot verdediging en patogene-reaksies, geidentifiseer is wat gelyktydig in beide *Lotus* en tamatie uitgedruk word. Deur ‘n klein aantal gene te identifiseer, wat betrokke is by groeistimulasie, kan die gene in die toekoms vir funksionele analieses gebruik word deur van keerkoppeling-genetika gebruik te maak. Daardeur sal meer insig verkry word in die molekulêre meganisme wat deur lumikroom as ‘n plantgroei stof veroorsaak word.

FOR MY MOTHER

Happiness is only real when shared

-Christopher Johnson McCandless-

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ABBREVIATIONS

BSA	Bovine serum albumin
bp	base pairs
e.g.	for example
FW	fresh weight
DIG	digoxigenin
DTT	dithiothreitol
g	gram
GAPDH	glycerladehyde-3-phosphate dehydrogenase
GS	glutamine synthetase
GC-MS	gas chromatography mass spectrometry
IEF	isoelectric focusing
kDa	kilo Dalton
LC-ESI-MS/MS	liquid chromatography electrospray ionization mass spectrometry/mass spectrometry
MALDI-MS	matrix-assisted laser desorption mass spectrometry
MS	Murashige and Skoog, i.e. Murashige, T and Skoog F (1962) A revised medium for rapid growth bioassays with tobacco tissue culture. Physiologia Plantarum 15:473 – 497
mRNA	messenger RNA
nM	nano molar
NADH	Nicotineamide adenine dinucleotide
PCR	polymerase chain reaction
PCA	principal component analysis
PVP	polyvinylpyrrolidone
RT	room temperature
rpm	revolutions per minute
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
UDP	uridine diphosphate
V	volt

CHAPTER 1

General Introduction

Plant growth is a complex, yet well-organized and coordinated process. Growth is commonly defined as the increase in the amount of living material, which is based on an increase in cell size and cell division. In plants, cell division occurs in specialized meristem regions such as the apices of primary roots and stems. As these regions are displaced distally by the cells they create, cells are left behind that cease division but continue to grow and therefore expand extensively. Balancing cell division and expansion with plant growth is evident in the relatively constant size of the cells at different growth rates caused by varying light conditions, water availability, solute concentration and other environmental factors (Granier *et al*, 2000; West *et al*, 2004; John and Qi, 2008). Growth only occurs in living cells which are metabolically active and involved in the synthesis of proteins, nucleic acids, lipids and carbohydrates at the expense of metabolic energy provided by photosynthesis and respiration. Photosynthesis drives plant growth where carbon (C) is assimilated in source leaves. The assimilated carbon is then exported, mostly as sucrose, to support the growth of sink organs like developing leaves and roots. In turn, more CO_2 is assimilated and other resources such as mineral nutrients and water is acquired (Stitt *et al*, 2007). Various parameters have been used to evaluate plant growth including fresh weight, dry weight, root to shoot ratio, shoot number or shoot length (Li *et al*, 1998; Leister *et al*, 1999). The growth rate and relative growth rate (RGR) are comprehensive traits of plants that characterize plant performance (McGraw and Garbutt, 1990). These parameters integrate morphological and physiological traits of plants (El-Lithy *et al*, 2004).

Growth can be seen as the integration of a wide variety of processes. These include morphological, physiological and genetic characteristics that change in response to an ever changing environment. Plant growth analysis is an essential step in the understanding of plant performance and productivity and may reveal different strategies of plants to cope with their changing environment (El-Lithy *et al*, 2004). How plants respond to changes in the environment on molecular and physiological levels can be investigated to further our understanding of general growth responses found in most plant species when confronted with

a changing environment. Information regarding these responses to challenges can be utilized to genetically engineer crops that can cope with these daily challenges in a better way.

Factors that influence plant growth

Plant growth ultimately depends upon changes in the environmental variables, such as temperature, light intensity and the availability of water and essential minerals (Hermans, *et al*, 2007). As plants are sessile organisms, they have to adapt to an array of abiotic and biotic factors to maintain optimum growth and respond flexibly to environmental challenges. Plants constantly sense changes in the environment and respond to various stress conditions such as nutrient deficiency, hypoxia, drought stress, heat stress and heavy metal stress. In response to stress, plants have strategies in place to adapt to these changes. These include changes in gene expression, enzyme activities and metabolite levels. Stress conditions can have both negative and positive effects on plants. Exposure to stress can lead to disruption of cellular and molecular processes that can boost the stress tolerance of the plant through induction of acclimation responses. Stress tolerance includes responses on morphological, physiological and biochemical levels, decreasing stress exposure, limit damage or facilitate repair of damage (Potters *et al*, 2007; Mittler, 2002). Plants seem to have a general response to stress called the "stress induced morphogenic response" (SIMR). This response appears to be carefully orchestrated and encompasses three components: a) inhibition of cell elongation, b) localized stimulation of cell division and c) alterations in cell differentiation status. Plant growth is redirected to diminish stress exposure e.g. phosphate starvation where the root system in *Arabidopsis thaliana* is altered which includes increased differentiation, increased lateral root formation and decreased root elongation (Williamson *et al.*, 2001; Potters *et al*, 2007). It is hypothesized that similar responses to stress conditions reflect common molecular processes such as increased reactive oxygen species (ROS) production and alterations in phytohormone transport and metabolism (Potters *et al*, 2007).

Plants tend to respond to nutrient shortage through biomass allocation. Nitrogen (N) and phosphorus (P) deficiencies cause increases in root biomass as carbohydrates are accumulated in the leaves and higher levels of carbon is allocated to the root, leading to an increase in the root:shoot ratio. These alterations therefore affect photosynthesis, carbohydrate partitioning and metabolism and alter root morphology (Hermans *et al*, 2007), illustrating the adaptive nature of plants. Thus, one can stipulate that stress influences various aspects of plant growth simultaneously.

Plant growth promoting substances and plant growth promoting bacteria have the ability to alter and exert beneficial effects on plant growth. In the rhizosphere, the region around the root, bacteria are abundantly present. Plant growth promoting rhizobacteria (PGPR) are beneficial bacteria that colonize the roots of plants and stimulate plant growth. PGPR are used as inoculants for biocontrol, biofertilization and phytostimulation and they can modulate plant growth by enhancing the availability of nutrients, inducing metabolic activities by phytohormones or by shifting the hormonal balance. Bacteria excrete chemical compounds that can stimulate and influence plant growth and development, whilst nutrients secreted by plant roots benefit the growth of rhizobacteria. In addition, they have the ability to induce defence mechanisms such as systemic acquired resistance (SAR) (Ping and Boland, 2004) and alleviate some stress conditions. Kohler *et al* (2008) have shown, that the potential use of PGPR as an inoculant can alleviate oxidative damage produced under water stress.

Rhizobia produce signal molecules called lipo-chitooligosaccharides (LCOs) during the establishment of rhizobia-legume nitrogen fixing symbiosis. These nodulation factors (Nod factors) are complex compounds and active at picomolar concentrations (Spaink, 1996). Apart from their function in the nodulation process, Nod factors have been shown to increase seed germination and plant growth (Souleimanov *et al*, 2002; Prithiviraj *et al*, 2003), including lateral root growth (Oláh *et al*, 2005). Other incidences have been reported where foliar application of LCOs increased the plants resistance to diseases, assist in overcoming temperature stress and lead to a reduction in yield losses of soybean plants under drought conditions (Duzan *et al*, 2005; Miransari *et al*, 2006; Atti *et al*, 2005). Supanjani *et al* (2006) showed that the addition of the Nod factor NodBj-V (C18:1 MeFuc) to soybean seedlings led to improved calcium uptake and growth.

Plant growth promoters modify or control specific biological processes in plants, which in turn alters the growth of the plant. A range of low molecular weight compounds have recently been identified that stimulate plant growth, but which do not fall into the usually recognized classes of plant hormones. These include compounds from plant growth promoting rhizobacteria e.g. lumichrome (Phillips *et al*, 1999), 2,3-butanediol (Ryu *et al*, 2004), aqueous smoke (Sparg *et al*, 2005), polyamines (Galston and Kaur-Sawhney, 1990), salicylic acid (Ping and Boland, 2004), humic substances (Clapp *et al*, 2001) and nitric oxide (Grün *et al*, 2006). A common characteristic is that these substances mainly function in very low concentrations and often high concentrations will inhibit growth. They have a complex mode of action and trigger other physiological processes such as induced systemic pathogen

resistance (Ping and Boland, 2004; Ryu *et al*, 2004) and seed germination (Flematti *et al*, 2004; Van Staden *et al*, 2004).

Although bacteria are known to affect fundamental processes in plant development, the mode of action remains unknown. More research has been conducted recently, where many diazotrophs, including rhizobia, have been shown to use chemical molecules to effect changes in plant development. One of these molecules is lumichrome. Lumichrome, which is a degradation product of riboflavin, was identified from culture filtrates of *Sinorhizobium meliloti* cells with the ability to stimulate plant growth (Phillips *et al*, 1999). Phillips *et al* (1999) showed that lumichrome enhances root respiration in alfalfa (*Medicago sativa*) plants, thereby generating more exogenous CO_2 upon which rhizobial growth is dependent. It improves the growth of alfalfa prior to the onset of nitrogen fixation and is attributed to an increase in net carbon assimilation.

The stimulatory effect of smoke and aqueous smoke solutions on plant growth and development, seed germination, seedling vigor, flowering and rooting have been proven by various groups. The active compound from smoke, the butenolide 3-methyl-2H-furo[2,3-c]pyran-2-one, has been isolated (Flematti *et al*, 2004; van Staden *et al*, 2004) and proven in various studies that it promotes plant growth (Sparg *et al*, 2005; van Staden *et al*, 2006; Daws *et al*, 2007; Kulkarni *et al*, 2007; Sóos *et al.*, 2009). The mode of action and the mechanism of plant growth stimulation by smoke are still unknown; however it can now be investigated since the active compound is known. It is quite interesting to note that the natural product strigol, which promotes the germination of the parasitic weed Striga, is active at similar concentrations as butenolide and contains a butenolide moiety, similar to that of the butenolide stemming from the aqueous smoke solution (Flematti *et al*, 2004).

Other important and sometimes not eminent growth regulators have been identified, which play vital roles in plant growth and development. Nitric oxide (NO) is a free radical involved in numerous and diverse cellular pathways in mammals (Torreilles, 2001). In recent years researchers established that NO plays a pivotal role in the plant kingdom as well, with involvement in plant growth and developmental processes as well as defence responses. Regarding NO's involvement in plant growth, the list is endless: vegetative growth processes of the shoot, cell division, xylem differentiation and leaf expansion (Grün *et al*, 2006).
Specifically, a central role for NO as a chemical signal involved in root growth and development and in the interaction of roots with plant growth promoting rhizobacteria

Azospirillum was presented by Molina-Favero *et al* (2008). Additionally, the participation of NO in a number of plant signaling pathways is well described (Grün *et al*, 2006).

Manipulation of plant growth

Alterations in plant growth are not only the result of stress conditions imposed on a plant or treatment with certain plant growth regulators, but other aspects too can alter plant growth. Researchers have used different approaches in manipulating plant growth to gain insight into how these fundamental processes take place. How then do we best manipulate the growth of a plant in order to understand their molecular and physiological responses in a better way and utilize this information for genetic engineering of more robust and productive crops?

Inducing a stress condition e.g. drought stress and therefore forcing the plant to adapt to these changes, is a way of gathering information about essential growth processes. One can analyze their morphological and molecular response to the specific stress condition through exploring changes in gene expression, protein expression, enzyme activities and metabolite levels in combination with e.g. photosynthesis measurements.

Other approaches include recombinant inbred lines (RIL) (Meyer *et al.*, 2007) or genetically modified plants that overexpress a specific gene of interest. Limami *et al* (1999) took both of these approaches, as they wanted to investigate the contribution of root cytosolic glutamine synthetase (GS) activity to plant biomass production. Firstly, glutamine synthetase was overexpressed in roots of transgenic plants, which led to decreases in plant biomass production. Secondly, the relationship between GS activity and biomass production was analyzed using a series of recombinant inbred lines issued from the crossing of two different *Lotus japonicus* ecotypes, Gifu and Funakura, which confirmed the negative relationship between GS and biomass production. Often researchers take a chemical genetics approach. This is where small molecules are used to change the way proteins function, thereby identifying which proteins regulate which biological processes and to understand in detail how they perform their biological function. Both forward and reverse chemical genetic approaches are possible, in agreement with classical genetic approaches. Forward chemical genetics involves the screening of synthetic molecules in cells or organisms for phenotypic changes, the selection of a molecule that induces the phenotype of interest and the eventual identification of the protein target of the small molecule. The ultimate goal of forward chemical genetic approaches is target identification, especially if the target is believed to be novel (Blackwell and Zhao, 2003). The effect of these small molecules on global gene

expression can be examined through DNA microarray analysis, which can assist in target identification (Southern, 2001). Reverse chemical genetics involves the overexpression of a protein target of interest, the screening of compound libraries for a ligand that modulates the function of the protein in a cellular or organismal context (Blackwell and Zhao, 2003).

Quintessentially, we took a forward chemical genetic approach in this project. Our goal was to manipulate plant growth by adding unconventional and novel growth promoting substances to different plant species in diverse environments and to investigate their response on molecular and physiological level. This would allow us to understand how the growth promotion is exerted; therefore clarify their mode of action.

To conclude, an overview of all the aims and outcomes of this study is presented in context of the various chapters:

The main aim of this study was to investigate the molecular mechanism of the plant growth promoting substance lumichrome. Firstly, basic growth studies were conducted to evaluate and assess the effects of lumichrome as a plant growth promoter on *Lotus japonicus* and *Solanum lycopersicon*. Large scale profiling of gene expression, proteins and metabolites of lumichrome treated and untreated plants were performed to identify central themes and components of the lumichrome induced growth promotion. This was completed for both *Lotus japonicus* and *Solanum lycopersicon* roots, as basic growth studies revealed that lumichrome significantly increased root biomass. To verify the importance of two proteins, more detailed analysis was completed utilizing northern blot analysis, western blot analysis and the measurement of enzyme activities. Completing the profiling in both species would allow the identification of key regulators of plant growth and development in response to lumichrome and shed more light on the mechanism, as the mode of action has not been elucidated. Through identifying a small number of genes involved in mediating the growth stimulation, these could be used to engineer crops for enhanced vigor and productivity in the future as well as provide more insight into the molecular mechanisms of plant growth promoting substances.

Chapter 1

General Introduction

The first chapter aims at giving an overview of what plant growth is, why it is important to study plant growth, the factors that influence plant growth and some interesting plant growth regulators aside from the classical phytohormones. Additionally, it includes methods on how researchers manipulate plant growth and consequently asking the question why plant growth manipulation is important to investigate. Lastly, the overall aim of the project is described and the content of the various chapters explained.

Chapter 2

Unraveling the mystery behind the plant growth promoting substance lumichrome: A compound originating from plant growth promoting rhizobacteria

The second chapter provides an overview of what PGPR are, the different classifications according to their function, their importance and beneficial effects on plant growth. Thereafter, the plant growth promoting substance lumichrome, which is a compound originating from *Sinorhizobium melliloti* bacteria, is reviewed. Lumichrome is described according to its origin, different functions in various organisms and photobiological characteristics. Moreover, its effect on plant growth is described, the common features of plants influenced by lumichrome are highlighted and a possible mode of action described.

Chapter 3

The Molecular Physiological Effects of the Plant Growth Regulator Lumichrome on *Lotus japonicus*

Aim: This chapter aims at determining the growth promoting effects of lumichrome on *Lotus japonicus*. Differentially expressed genes and proteins as well as altered metabolite levels in *Lotus japonicus* roots as a result of lumichrome treatment are investigated. In addition, a more targeted analysis involving northern blot, western blot and the measurement of enzyme activities aims at investigating the importance of specific genes and proteins.

Outcome: Plant growth analysis revealed that at nanomolar concentrations, lumichrome significantly increased root growth in *Lotus japonicus*. Results of transcript profiling in the roots showed that the top three categories of differential gene expression were RNA regulation of transcription, signaling and stress and defence. Two dimensional (2-D) gel electrophoresis resulted in the identification of five proteins that were differentially expressed,

all showing less abundant levels of the specific protein in lumichrome treated roots. In the metabolite analysis, lumichrome treated roots showed signs of nitrogen deficiency and oxidative stress. The targeted analysis of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) showed that this protein might be under post-translational modification and the detailed analysis of glutamine synthetase (GS1) demonstrated that with the presence of five isoenzymes, a complex mechanism appears to be at work.

Chapter 4

Lumichrome promotes growth of tomato (*Solanum lycopersicum*) roots and induces the expression of orthologous defence-related genes across species

Aim: This chapter aims at determining the growth promoting effects of lumichrome on *Solanum lycopersicum*. Differentially expressed genes and proteins as well as altered metabolite levels in *Solanum lycopersicum* roots as a result of lumichrome treatment are investigated. In addition, a more targeted analysis involving northern blot analysis and the measurement of enzyme activities aims at investigating the importance of GAPDH in the lumichrome mediated growth promotion.

Outcome: Plant growth analysis revealed that lumichrome significantly increased root growth at nanomolar concentration in *Solanum lycopersicum*. RNA regulation of transcription, protein synthesis/degradation/modification and stress and defence were the top three categories that showed the most changes regarding differential gene expression in tomato roots. 2-D gel electrophoresis resulted in the identification of five proteins that were differentially expressed. Four proteins showed less abundant levels in lumichrome treated roots. Interestingly, three of these proteins are involved in glycolysis and one candidate was identified as GAPDH, which too was down-regulated in *Lotus japonicus* roots and targeted analysis showed the involvement of post-translational modification. The metabolite analysis revealed only minor changes in metabolite levels. Ultimately, lumichrome seems to induce similar changes in gene expression in both lumichrome treated *Lotus* and tomato plants, relating to defence and pathogen responses. The effects of lumichrome was ultimately assesed in the cross species validation between tomato and *Lotus* roots, where six candidate genes were identified that seem to play a pivotal role in the lumichrome induced growth promotion.

Chapter 5

General Discussion and Conclusion

The observations and discussions from the previous chapters are integrated and discussed.

CHAPTER 2

Unraveling the mystery behind the plant growth promoting substance lumichrome: A compound originating from plant growth promoting rhizobacteria

In the rhizosphere, bacteria are abundantly present and often organized in microcolonies. Among these are rhizobacteria that beneficially influence plants through growth promotion in a direct or indirect way. These so-called plant growth promoting rhizobacteria can be classified according to their function.

Firstly, "biofertilizers" can fix atmospheric nitrogen which consequently can be used by the plant to improve its growth when the amount of nitrogen is limited in the soil (Bloemberg and Lugtenberg, 2001). Other PGPR biofertilizers influence the availability of phosphate by secreting phosphatases for mineralization of organic phosphorus or by releasing organic acids for the solubilization of inorganic phosphates (Rodríguez and Fraga, 1999). Another example of biofertilization would be the release of siderophores that chelate iron and make it available to the roots of the plant (Bloemberg and Lugtenberg, 2001).

Secondly, "phytostimulators" can directly promote plant growth, usually through the production of hormones or by promoting nutrition (Bloemberg and Lugtenberg, 2001). Some PGPR have the ability to produce auxin, cytokinin and gibberellin, octadecanoids and compounds that mimic the action of jasmonates. Others are known to control the biosynthesis of ethylene via 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, which reduces the availability of the ACC pool required for ethylene biosynthesis. This is important to note, as ethylene often acts either synergistically or antagonistically with other plant hormones. Phytostimulation particularly pays off with a balanced network of plant hormones or hormone-like compounds that directly promote root growth e.g. in the case of *Azospirillium* where it produces auxins, cytokinins and gibberellins that stimulate root formation. Clearly, enhanced root formation means increased capacity to take up nutrients. This eventually leads to crop improvement (Ping and Boland, 2004).

Lastly, "biocontrol agents" are capable of protecting plants from infection by phyto-pathogenic organisms by inducing systemic resistance (ISR) or systemic acquired resistance (SAR) in plants (Bloemberg en Lugtenberg, 2001; Glick, 1995). Bacterial determinants that induce ISR include siderophores, the O-antigen of lipopolysaccharides and salicylate, which mediates SAR. Mostly, both ISR and SAR are activated simultaneously, ISR specifically when the plant is challenged by pathogenic organisms. Both though have independent signaling pathways (Bloemberg en Lugtenberg, 2001; Ping and Boland, 2004).

Various studies have been conducted, demonstrating the beneficial effects of PGPR. Specifically regarding Nod factors, which are lipo-chito-oligosaccharides (LCOs) and are generally composed of three to five 1-4β linked acetyl glucosamine residues with the N-acetyl group of the terminal non-reducing en replaced by an acyl chain. However, variations in this basic structure are possible as each of the rhizobia produces a specific set of Nod factors. Nod factors as well as synthetic LCOs are known to affect a number of host physiological processes in plants (Souleimanov *et al,* 2002). As a primary target, the root is the organ that shows the first stimulating effects of PGPR. Field experiments with *Azospirilla* inoculated crops showed significant increases in yield, which was accompanied by more efficient mineral and water uptake, positively changing the growth dynamics and morphology of the roots. The positive effects that *Azospirillum* has on plant growth, whether under normal - or during stressful conditions, rely on molecular mechanisms that are very poorly understood. Whatever the type of relationship that occurs between plants and rhizobacteria, the mechanisms and signals that enable the roots to interpret the signals that they receive from the rhizosphere and how exactly it leads to growth promotion, are largely unknown (Molina-Favero *et al,* 2008). It is known that *Azospirillum* can produce nitric oxide (NO) at low O_2 pressure by denitrification (Hartmann and Zimmer, 1994) and NO in turn functions as a signal molecule in the indole 3-acetic acid (IAA)-induced signaling cascade leading to the formation of adventitious roots, lateral roots and the formation of root hairs (Molina-Favero *et al*, 2008). Therefore NO might play a role in the root growth promoted by *Azospirillum*.

Rhizosphere compounds supplied to roots can alter physiological functions in plants through affecting stomatal functioning and transpiration (Joseph and Phillips, 2003). Following the foliar application of the Nod factor [Nod Bj V ($C_{18:1}$, MeFuc)], Souleimanov *et al* (2002) showed increased growth in corn and soybean and Khan *et al* (2008) illustrated increases in photosynthetic rates. It is important to note that LCO stimulates growth in non-leguminous plants as well, such as carrots and tomato and when added to a carrot cell culture, caused

increased somatic cell embryo formation in similar ways to auxin and cytokinin (De Jong *et al*, 1993; Dyachok *et al*, 2000)

Another study examined the effects of inoculation of the PGPR *Pseudomonas mendocina* on lettuce plants affected by water stress. Drought stress limits plant growth and production and may cause damage to cells through the formation of reactive oxygen species (ROS) such as superoxide radicals and H_2O_2. The PGPR had a positive effect on reactive oxygen metabolism, stimulating the activities of antioxidant enzymes and increasing proline accumulation under severe drought stress. Therefore, this provides evidence of the contribution of a PGPR to the development of mechanisms to alleviate the oxidative damage produced in plants under water-shortage conditions (Kohler *et al*, 2008).

Another study investigated the response of soybean under chronic water deficit to LCO application during flowering and pod filling. At medium water stress levels, LCO treatment had positive effects on the growth pattern of soybean. The LCO treatment affected overall plant physiology through an increase in the photosynthetic rate, increase in flower and pod numbers and accelerated leaf senescence. With sufficient water supply and severe water deficit, LCO treatment did not have any significant effect. The common stress level observed in standard farm-field conditions is medium water stress, thus LCO treatment could be a way of reducing negative drought stress effects in plants such as soybean and enhance its water use efficiency. One can harness these molecules for improvement of crop production under water scarcity ultimately augmenting the world food production (Atti *et al*, 2005).

Through powerful signal molecules, rhizobacteria affect fundamental processes in plants. In recent years, a number of novel rhizobial molecules have been identified that positively effect plant growth and development. From culture filtrates of *Sinorhizobium meliloti* cells, Phillips *et al* (1999) identified lumichrome as another rhizosphere signal molecule with the ability to promote plant growth. Lumichrome is a common breakdown product of riboflavin and produced by both chemical and biological factors. In the presence of light through a photochemical-induced cleavage of the ribityl groups under neutral or acidic conditions, riboflavin is converted to lumichrome (Yagi, 1962). Additionally, *Pseudomonas* bacteria enzymatically degrade riboflavin to lumichrome, thus light is not always required as it is not present in the natural rhizosphere environment (Yanagita and Foster, 1956).

In order to comprehend how lumichrome stimulates plant growth, one has to examine the characteristics of the compound itself. This might provide clues as to how exactly it exerts its function through certain signaling pathways. As lumichrome is the degradation product of riboflavin, it is important to explore the biological functions and characteristics of flavins (10-alkyl-7,8-dimethylisoalloxazines). Flavins are used as prosthetic groups by flavoproteins. The photoreactions of flavins have recently been of great interest due to the biological relevance of these compounds (Porcal *et al,* 2003). Flavins are involved in redox reactions and in the sensing of blue or ultraviolet light. In cryptochromes, flavin chromophores mediate flowering and daily light/dark cycles in plants, in phototropins they regulate phototropism and in photolyases they are involved in DNA repair (Meissner *et al,* 2007). Another interesting characteristic of alloxazines, is that they act as a ligand for selective binding to adenine opposite asbasic (AP) sites in DNA duplexes. Lumichrome, however, shows a clear selectivity for thymine over other nucleobases. Therefore, lumichrome might have a direct effect on gene expression. As riboflavin is one of the most important members of this group, its degradation product lumichrome (7,8-dimethylalloxazine) is of great importance too as they are structural analogues. Lumichrome is also generally found in biological material associated with flavins and may participate in biological processes. Flavins are commonly applied photosensitizers. The photochemical action of a sensitizer towards oxygen generally refers to electron and energy transfer, thereby yielding the hydroperoxyl/superoxide ion ($HO^{\bullet}_2/O^{\bullet}_2^{-}$) radical and singlet molecular oxygen O_2 ($^1\Delta_g$), respectively (Görner, 2007). Therefore, lumichrome acts as a photosensitizer, which means that it generates singlet oxygen when exposed to light. The importance of riboflavin and lumichrome was demonstrated when the photosensitizing effect of riboflavin, lumiflavin and lumichrome was tested on the generation of volatiles in soy milk. It has been reported in previous occasions, that singlet oxygen was involved in the flavour deterioration of soy milk and whole fat cow milk when supplemented with riboflavin under light (Huang, *et al,* 2004; Lee, 2002). Riboflavin is not stable in the presence of light and is quickly degraded to lumichrome and lumiflavin, which is very much dependant on the pH of the solution. Riboflavin, lumiflavin and lumichrome were found to act as photosensitizers to form singlet oxygen. The singlet oxygen formed could react with the lipid and protein components in soy milk, causing flavour deteriorations (Huang *et al,* 2006). Therefore, lumichrome can be seen as a good and efficient photosensitizer of singlet oxygen. The primarily non-toxic lumichrome was identified to be efficient in transferring excitation energy to substrates (photosensitization type 1) and oxygen (photosensitization type 2) and thus exerting a "secondary toxic" effect (Grininger *et al,*

2006). This characteristic may possibly be very important in plant growth and development especially in redox reactions, photosynthesis and oxidative stress.

To understand the mechanism of how lumichrome promotes plant growth, it might be interesting to look at what is known about lumichrome binding proteins. Dodecins are a novel family of flavin-binding proteins and thus far, the smallest known flavoprotein with only 68 amino acids. The proteins were first discovered in *Halobacterium salinarum* (Grininger *et al,* 2006) and apart from haloarchaea, are found in many eubacterial genomes as 16% of all completely sequenced eubacteria possess dodecin encoding genes (Meissner *et al,* 2007). Grininger *et al* (2006) found that the dodecins of *H. salinarum* have a high binding affinity for lumichrome and lumiflavin. They postulated that these dodecins might serve as a waste-trapping device, protecting the cellular environment from high amounts of phototoxic lumichromes, generated by the photoinduced degradation of riboflavin. In contrast, Meissner *et al* (2007) found that the dodecins from *Thermus thermophilus* binds all flavins with similar binding constants. They proposed a scenario for the biological function of dodecins might be that of a flavin trap, functioning when the cytosolic concentration of free flavin increases, for example after heat shock or flavin release from denatured flavoproteins.

Previous studies have shown that *Sinorhizobium meliloti* increases CO_2 availability by enhancing root respiration in alfalfa (Volpin and Phillips, 1998; Phillips *et al,* 1999). After various experiments, Phillips *et al* (1999) could show that lumichrome was the active compound and suggested that it represents a previously unrecognized mutualistic signal molecule in the *Sinorhizobium*-alfalfa association. Various studies on lumichrome have proven its growth promoting effect in the interaction with plants but the mechanism and mode of action is still unknown. Exploring the common characteristics displayed by plants treated with lumichrome in various studies might aid in postulating a possible mechanism. Plants treated with lumichrome showed increases in biomass (Phillips *et al,* 1999; Matiru and Dakora, 2005a), influence on photosynthetic rates (Matiru and Dakora, 2005b; Khan *et al,* 2008), increases in root respiration (Volpin and Phillips, 1998; Phillips *et al,* 1999; Matiru and Dakora, 2005b), changes in the stomatal conductance (Joseph and Phillips, 2003; Matiru and Dakora, 2005b) and transpiration (Joseph and Phillips, 2003; Matiru and Dakora, 2005b; Khan *et al,* 2008). Additionally, the growth promotion was not age specific and the presence of this signal molecule in high concentrations in the rhizosphere had an inhibitory effect on plant growth. These characteristics were species dependent and varied in plants that responded to lumichrome.

Diverse studies have proven that lumichrome is a plant growth promoting substance. Phillips *et al* (1999) showed that by applying 5 nM lumichrome to young alfalfa roots, the plant growth increased by 8% after 12 days. Soaking the seeds in 5 nM lumichrome before germination, increased growth by 18% over the same period. In both cases, the growth enhancement was significant only in the shoot. To investigate whether this growth response was unique to alfalfa, Matiru and Dakora (2005a) assessed the stimulatory role of lumichrome on legume and cereal seedlings. At nanomolar concentrations, lumichrome elicited growth promotion in cowpea, soybean, sorghum, millet and maize, but not in common bean, Bambara groundnut and Sudan grass. The growth promotion was not age specific. Khan *et al* (2008) applied lumichrome to soybean and found an increase in leaf area, shoot dry mass and total dry mass relative to control plants. Corn, however, did not show any significant differences compared to the control.

Besides growth studies (Matiru and Dakora, 2005a), Matiru and Dakora (2005b) performed some experiments where they measured root respiration, stomatal conductance and leaf transpiration in lumichrome treated legumes and cereals. Lumichrome significantly increased root respiration in maize. However, lumichrome application to lupin decreased root respiration and did not affect cowpea, soybean, Bambara groundnut, pea or sorghum. The stomatal conductance was decreased in most of the plants that were treated with lumichrome, except for cowpea and lupin. Consequently, an increase in transpiration was observed where stomatal conductance was increased. Photosynthesis was decreased in cowpea and sorghum plants treated with lumichrome. In addition, Matiru and Dakora (2005b) assessed whether lumichrome applied to roots of monocots and dicots is transported via xylem and accumulated in the shoots. With HPLC analysis, they established the presence of lumichrome in the xylem stream of plants as well as demonstrated its accumulation in leaves. In soybean, the increased lumichrome concentration in the xylem stream corresponded to the increased accumulation of lumichrome in leaves. Furthermore, there were differences between soybean and cowpea in that a higher concentration of lumichrome was found in the xylem of soybean than in cowpea, reflected in the more dramatic developmental changes observed in soybean.

Khan *et al* (2008) measured the photosynthetic rates, stomatal conductance as well as the leaf internal CO_2 values of corn and soybean plants treated with foliar application of lumichrome. The photosynthetic rates of corn and soybean increased upon lumichrome treatment, as well as the stomatal conductance and transpiration rates. Interestingly, in previous studies it has been shown that soil organisms increase net photosynthesis in diverse plant species (Meharg

and Killham, 1991; Merbach and Ruppel, 1992). Compared to the control, the leaf internal CO_2 values of corn were higher in lumichrome treated plants but only differed from the control on day two. Soybean intercellular CO_2 were not different from the untreated controls. These findings strongly suggest that lumichrome mediated growth promotion seems to be species specific and each plant responds to lumichrome in its own unique way, which makes the task of unraveling the mode of action even more challenging.

The mode of action regarding lumichrome is still unknown. The benefit to rhizobacteria of enhancing root respiration with lumichrome is the increased availability of CO_2 which is a growth requirement for rhizobia (Lowe and Evans, 1962). Increases in root respiration require an increased flow of carbon substrates to support the additional respiration. This in turn enhances the root exudation of plant compounds beneficial to bacteria. With Rhizobia-legume interactions, the legume profits from nitrogen (N) compounds supplied by the bacteria in the mature root but what happens in the case of non-leguminous plants where there is no symbiosis in the classical way? Matiru and Dakora (2005a) suggested that lumichrome supply probably altered assimilate partitioning resulting in increased root growth. Moreover, the activity of lumichrome is similar to classical phytohormones such as abscisic acid (ABA), promoting root growth at low concentrations, and inhibiting it at higher levels (Aspinall *et al*, 1967). Possibly, the fact that lumichrome is transported in the xylem and accumulated in the shoots may point to direct elicitation of cell division, cell expansion and extensibility which leads to increased growth (Matiru and Dakora, 2005b). There is a strong possibility that lumichrome acts synergistically or antagonistically with phytohormones such as cytokinin, gibberellic acid (GA) or auxin to exert the growth promotion. A few questions arise as to how lumichrome exactly causes the growth promoting effect: does lumichrome act primarily through the root or the shoot? What are the molecular mechanisms involved, that is, the signaling events responsible for the growth promotion? Do plants possess dodecin-like proteins that bind to lumichrome and if so, do they play a pivotal role in the mechanism of plant growth promotion? Are there similarities between Nod-factor signaling events and signaling events mediated by lumichrome?

The aim of this project is to answer some of these questions, as it is vital that we have a better understanding of plant-microbe interactions that influence plant growth. Information obtained through this and other studies must aid us in the agricultural application of these plant growth promoting substances, which potentially effect growth and yield of crop plants. Further, investigating the physiological and molecular effects of these and similar compounds can

assist us in understanding their mode of action, find a possible common mechanism and use them as bioregulators in future agricultural production. Numerous growth- and physiology studies have proven the growth promoting effect of lumichrome, but this is the first study to our knowledge investigating changes in gene expression, proteins and metabolites as a result of lumichrome application. Consequently, the outcome of this study will offer more insight into the mode of action of lumichrome mediated growth promotion.

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CHAPTER 3

The Molecular Physiological Effects of the Plant Growth Regulator Lumichrome on *Lotus japonicus**

ABSTRACT

The response of *Lotus japonicus* roots to lumichrome on gene expression, protein and metabolite levels was evaluated. On the gene expression level, DNA microarrays revealed increases in the gene expression of the functional categories stress and defence, signaling and RNA regulation of transcription. Remarkably, many of the genes in the categories signaling and RNA regulation of transcription are also implicated in abiotic and biotic stress responses, indicating that lumichrome is perceived as a stress signal. The proteomic analysis resulted in the identification of five proteins that were present in lower abundance in lumichrome treated roots. A more targeted analysis of GS1 and GAPDH was performed to confirm the relevance and importance in the lumichrome mediated growth promotion. Contradictory to the proteomic results, northern blot analysis indicated an increase in transcript levels of GS1 and GAPDH. The microarray data confirmed the result, however, only for GS1. Enzyme activities for both GS1 and GAPDH showed no significant difference between lumichrome treated and untreated roots. Moreover, western blot analysis for GS1 could not detect significant differences in protein levels. Therefore, one can conclude that GAPDH seem to be under post-translational modification, however, further investigation is needed to confirm this statement. With GS1, further analysis is needed, as multiple isoenzymes exist for GS1 and the protein analyzed with 2-DE could be one of the isoenzymes showing differential expression. However, both seem to play a pivotal role in lumichrome mediated growth promotion although a complex mechanism appears to be at work. In treated plants, most of the metabolite changes occurred in the amino acid pool and point to nitrogen deficiency and oxidative stress.

Keywords

Plant growth regulator, lumichrome, transcriptomics, proteomics, metabolomics

* TO BE SUBMITTED FOR PUBLICATION

INTRODUCTION

Plant growth is a coordinated process where primary metabolism provides the power and building blocks for plants to grow. Plant hormones regulate the rate of growth of individual parts for instance the shoot or root and integrate these parts to produce the form we recognize as a plant. Classically, only six groups of hormones were thought to regulate plant growth and development (abscisic acid, auxins, brassinosteroids, cytokinins, ethylene and gibberellins) but more recent studies have shown that other interesting low molecular weight compounds have recently been identified as plant growth promoters that are undeniably out of the ordinary, do not fall into the usually recognized classes of plant hormones and indeed exert a growth promoting effect. These include phenolics, polyamines, methyl jasmonates, aqueous smoke solutions and compounds originating from plant growth promoting rhizobacteria.

Plant growth promotion by soil microorganisms is far from uncommon and can be considered part of a continuum in which interactions between plants and microorganisms range from deleterious, that is pathogens, to beneficial e.g. plant growth promoting rhizobacteria. The plant growth promoting rhizobacteria *Sinorhizobium meliloti* increase CO_2 availability by enhancing alfalfa root respiration with an extracellular compound (Volpin and Phillips, 1998; Phillips *et al.*, 1999). The compound was identified as lumichrome, a previously unrecognized rhizosphere signal molecule that has been shown to promote plant growth in various studies. Phillips *et al.* (1999) showed that by applying 5 nM lumichrome to young alfalfa roots, the plant growth increased by 8% compared to untreated controls after 12 days. The growth enhancement was evident only in the shoot. Matiru and Dakora (2005a) assessed the growth impact of lumichrome on legumes and cereal seedlings. At nanomolar concentrations, lumichrome elicited growth promotion in cowpea, soybean, sorghum, millet and maize, but not in common bean, Bambara groundnut and Sudan grass. Khan *et al.* (2008) applied lumichrome to soybean and found an increase in leaf area, shoot dry mass and total dry mass relative to control plants.

The mechanism through which lumichrome increases plant growth remains to be clarified. *S. meliloti* requires exogenous CO_2 for growth and may benefit directly from enhanced root respiration triggered by lumichrome. Soil microorganisms enhance net photosynthesis in diverse plant species (Merbach and Ruppel, 1992) and lumichrome could contribute to this effect. Khan *et al.* (2008) observed an increase in photosynthetic rates in corn and soybean. At this stage, it is not known whether lumichrome acts primarily through the shoot or the root.

As the compound was applied to roots in most of the experiments, one would postulate an initial effect on root respiration. Matiru and Dakora (2005b) showed that supplying soybean and cowpea roots with their respective homologous rhizobia and/or purified lumichrome increased the concentration of this molecule in xylem sap and leaf extracts. It is therefore transported in the xylem to the shoot, but what are the signaling events in the leaf accompanying the exerted promoting effects on plant growth?

The aim of this study was to establish the growth promoting effect of lumichrome on *Lotus japonicus* plants and to gain insight into the mechanism of lumichrome mediated growth promotion. *Lotus japonicus* was used as a model system as the growth promoting effects has not been tested on *Lotus* and it has become a popular model plant for plant biochemistry and molecular biology purposes. It has a relatively small genome (471.2 Mb), has a fast generation time (2 months) producing numerous seeds, is self-fertile and in contrast to other legumes, is a diploid with six chromosomes in the haploid genome (Sato and Tabata, 2005). Self-fertility facilitates the production of homozygous lines, which makes it practical for genetic studies. *Lotus japonicus* is related to the birdfoot trefoil (*Lotus corniculatus*), which together with, *Lotus uliginosus* and *Lotus tenius* (Swanson *et al.*, 1990) are important forage legumes for beef and sheep production.

Utilizing DNA microarrays, differential gene expression in the roots of *Lotus japonicus* was investigated at the mRNA level as the growth promotion was most prominent and significant in the roots. Secondly, on the protein level 2-D gel electrophoresis (NEPGHE) was exploited to look at differential protein expression between lumichrome treated and untreated control plants, therefore identifying proteins that play a pivotal role in the growth promotion. Lastly, a metabolite profile was compiled of lumichrome treated and untreated roots to assess the impact of lumichrome on the metabolome of *Lotus japonicus*. Additionally, a more targeted approach was taken with northern blot analysis, investigating differential gene expression of specific genes based on the proteomic results and western blot analysis of a specific protein, to confirm the results obtained from 2-DE analysis. In integrating the "omics" data, that is transcriptomics, proteomics and metabolomics, we can begin to get a better understanding of plant responses to lumichrome and in general, apply the knowledge gained to fundamental processes such as plant growth.

MATERIAL AND METHODS

Growth studies and plant material

Lotus japonicus seeds were scarified with 100% sulfuric acid for 8 minutes and washed five times with sterile distilled water. Seeds were sown in 0.5 l pots containing a mixture of 1:3 sand and vermiculite. Pots were initially covered with transparent plastic wrap for approximately six days. Plants were watered every second day with half-strength Hoagland solution containing 1 mM NH_4NO_3 and the effects of lumichrome on plant growth tested by adjusting the solution to contain 0 nM and 5 nM lumichrome, as described by Phillips *et al.* (1999). Plants were grown in a growth chamber with a PPFD (photosynthetic photon-flux density) of ± 300 μmol m^{-2} s^{-1}, where a 16 h photoperiod was maintained. The temperature was 21 ± 2°C with a relative humidity of approximately 50%. Plants were harvested at the onset of flowering and ground to a fine powder in liquid nitrogen. Samples were stored at -80°C until used. For the tissue culture experiments, the seeds were sterilized and put on half-strength Murashige and Skoog (Murashige and Skoog, 1962) medium (1/2 strength MS) supplied with 0.8% agar to germinate. After germination, the seedlings were transferred to ½ strength MS supplied with 0.8% agar and 0 nM and 5 nM lumichrome.

Transcript profiling

RNA extraction

Total RNA was isolated according to a modified CTAB method of Chang *et al.* (1993). Two hundred miligrams of root material was extracted with 1.2 ml of CTAB buffer (2 % (w/v) CTAB, 2% (w/v) PVP, 100 mM Tris-HCl (pH 8.0), 25 mM EDTA, 2 M NaCl, 0.5 g/L spermidine) vortexed immediately and incubated at 65°C for 20 minutes. Samples were centrifuged at 13 000 rpm for 10 minutes and the supernatant transferred to a new tube. A chloroform:isoamylalcohol (24:1; v/v) extraction was conducted twice – after each addition of C:I, the samples were vortexed for 30 seconds and then centrifuged for 15 minutes at 13 000 rpm. The RNA was precipitated overnight at 4°C by adding 8 M $LiCl_2$ to a final concentration of 2 M. The RNA was pelleted by centrifugation at 13 000 rpm for 60 minutes at 4°C. The pellet was resuspended in 50 μl of milliQ water. RNA was quantified spectrophotometrically and the quality visualized by loading five μg on an ethidium bromide stained 1.2% (w/v) agarose gel. RNA was treated with DNase I (Promega, Madison, WI, USA) according to the

manufacturers' instructions followed by precipitation with 5µl 3 M sodium acetate (pH 5.5) and 200 µl 100% ethanol at -20°C overnight. After the RNA was pelleted at 13 000 rpm for 40 minutes at 4°C, it was washed with 70% (v/v) ethanol and redissolved in 45 µl deionized water. The RNA was then column cleaned with the RNA MinElute Cleanup Kit (Qiagen) according to the manufacturers' instructions. The quality of each of the RNA samples was determined by evaluating the relative amounts of 28S and 18S ribosomal peaks using a Bioanalyzer (Agilent Technologies, Palo Alto, CA) and quantified on a Nanodrop (Thermo Fisher Scientific, USA).

Gene chip microarray experiments and data analysis

Ten µg of total RNA was used as template for amplification. Probe labeling, chip hybridization and scanning were performed according to the manufacturers' instructions (Affymetrix, Santa Clara, CA). Three biological replicates per treatment were hybridized independently to the Affymetrix GeneChip® *Lotus* Genome Array. Data was normalized using robust multi-chip average (RMA) as described in Irizarry *et al.* (2003). Differentially expressed genes were selected using Associative Analysis as described by Dozmorov and Centola (2003). With this method, a group of stable expressed genes was identified from the control samples and designated as the "Reference Group" to measure internal noise. Type I family-wise error rate was reduced using a Bonferroni Correction p value of 0.05/N, where N represents the 61 460 genes presented on the chip. Genes that showed 2-fold or greater change in transcript level, between samples, were selected as differentially expressed.

Proteomic analysis

Protein extraction

Approximately 100 mg of root material, stemming from each of the six treated and untreated control plants respectively, was weighed. Thereafter samples were paired together, resulting in three lumichrome treated and three untreated control samples containing 200 mg each. Samples were then lyophilized and subsequently used for protein extraction. For the protein extraction, 200 mg of root material was rapidly homogenized in liquid nitrogen with a mortar and pestle with sand. Proteins were extracted by adding 6 volumes of AP buffer (WITA GmbH, Teltow, Germany) and 0.02 volumes of PP buffer (WITA GmbH, Teltow, Germany) and incubated at 20°C for 20 minutes. Samples were centrifuged for 20 minutes at 13 000 rpm and the supernatant transferred to a new tube. Proteins were quantified using the Bradford

method (Bradford, 1976) using BSA as a standard. The quality of the extracted proteins was verified with one dimension 12% (w/v) SDS-PAGE according to Laemmli (1970).

First dimension (Isoelectric Focusing) and second dimension (SDS PAGE) gel electrophoresis

The subsequent protein separation was performed with two-dimensional, non-equilibrium pH-gradient electrophoresis (NEPHGE) as previously described by O'Farrell *et al.*, (1977). For the first dimension, 400 µg of protein was separated in 1.5 mm x 24.5 cm tube gels. Electrophoresis was carried out at 100 V for 1h, 200 V for 1h, 400 V for 17.5h, 600 V for 1h, 1000 V for 30 minuntes, 1500 V for 10 minutes and 2000 V for 5 minutes. SDS PAGE (15%) in the second dimension was performed (24 cm x 32 cm) with the electrophoresis carried out at 65 mA for 15 minutes and 55 mA for 6.5h. After electrophoresis, proteins were visualized with silver staining the gels as described by Celis *et al.* 1994.

Image acquisition and analysis

In the first step, 300 dpi 16-bit grey scale TIF images of gels were obtained using a Microtek XL 9800 Scanner (Mikrotek Scanners, Australia). These TIF – images were reduced in size by a factor of two in order to reduce the file size and subsequently imported into Melanie 2D – Gel Evaluation Software (Geneva Bioinformatics (GeneBio) SA, Geneva, Switzerland). The software identified a list of 233 variant spot groups and to validate these suggestions, the spots were sorted (based on the Student *t*-test at 95% significance level) according to the following criteria: spot detection quality, matching quality, spot position and spot intensity. Spot detection quality and the matching quality determine the quality of spot quantification. Spot position and spot intensity determine the likelihood of successful protein identification. In order to obtain valid candidate groups for mass spectrometric protein identification, the spot groups initially suggested by the software were checked for: A) Matching and spot detection errors. If errors were found which diminish the significance of the suggested change, the candidate group was rejected. If such errors were found which after removal would lead to a similar or better significance, the candidate group was still included. B) The size of the change should be at least 30%. Spot candidates meeting these criteria obtained their final priority according to spot intensity. Only spot groups which contain spots of > 0.1% Vol were suggested as nano-LC-ESI-MS/MS candidates. Selected spots were manually excised, digested with trypsin and analysed by MALDI-MS (two spots) or nano-LC-ESI-MS/MS (three spots).

Metabolite profiling

Primary metabolites were extracted and analysed as described by Roessner *et al.* (2001) optimised for tomato following the method of Roessner-Tunali *et al.* (2003). Gas chromatography coupled to electron impact ionization/time-of-flight mass spectrometry (GC/EI TOF-MS) was performed using an Agilent 6890N24 gas chromatograph (Agilent Technologies, Palo Alto, CA) with split or splitless injection connected to a Pegasus III time-of-flight mass spectrometer (LECO Instrumente GmbH, USA) run and evaluated according to Erban *et al.*, (2007) using TagFinder 4.0 software (Luedemann *et al.*, 2008). Chromatograms and mass spectra were evaluated using Chroma TOF 1.6 and TagFinder 4.0 software (Luedemann *et al.*, 2008). The peak height representing arbitrary mass spectral ion currents was normalized using the sample fresh weight and ribitol content for internal standardization. Metabolites were identified using NIST05 software and the mass spectral and retention time index (RI) collection of the Golm metabolome database (Kopka *et al.*, 2005; Schauer *et al.*, 2005). Significance levels of metabolite changes between lumichrome treated and control roots were evaluated by Student t-test ($P \leq 0.05$). Principal component analysis (PCA) was completed with Statistica 8 © StatSoft.

Northern blot analysis

Total RNA was isolated according to a modified CTAB method of Chang *et al.* (1993) (see transcript profiling). cDNA was reverse transcribed from total RNA using SuperScript III Reverse Transcriptase (Invitrogen corporation, Carlsbad, California, USA) according to the manufacturer's instructions. PCR amplification of the glyceraldehyde-3-phosphate dehydrogenase gene was achieved using 1 unit Taq DNA polymerase, 0.5 mM $MgCl_2$, 0.2 mM dNTP's, 1 x buffer, 0.2 µM Forward primer (ATGGGCAAGATCAAGATCGG), 0.2 µM Reverse primer (CACAGTAGGAACACGGAATG) and 1 µl cDNA reaction template in a final volume of 50 µl. PCR conditions were as follows: 5 minutes denaturation at 94°C; 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute and 30 seconds; final elongation step at 72°C for 7 minutes. PCR amplification of the glutamine synthetase gene was achieved using 1 unit Taq DNA polymerase, 1.5 mM $MgCl_2$, 0.2 mM dNTP's, 0.2 µM Forward primer (ATGTCGCTGCTTTCAGATCT), 0.2 µM Reverse primer (TCATGGTTTCCAGAGAATGG), 1 x buffer and 1 µl cDNA reaction template in a final volume of 50 µl. PCR conditions were as follows: 5 minutes denaturation at 94°C; 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute and 30 seconds; final elongation step at 72°C for 7 minutes. All the primers were obtained from Inqaba Biotec. (Pretoria, South Africa). The resulting amplicon of 720 bp was separated on a 1% (w/v)

agarose gel, the band excised and purified with the PCR purification Kit according to manufacturer's instructions (Qiagen, www.qiagen.com). The fragment was subjected to restriction digest to verify that the right fragment was amplified. For additional verification, the amplicon was cloned into pGEM®-T Easy (Promega, Madison, WI, USA) and sequenced. The resulting amplicons of 720 bp and 1070 bp, respectively were separated on a 1% (w/v) agarose gel, the band excised and purified. The purified amplicon was used for PCR DIG labeling. The fragment was subjected to restriction digest with *Bgl* II/*Eco* RI to verify that the right fragment was amplified. PCR labeling of the probe was achieved by using the PCR DIG Probe Synthesis Kit according the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). The probe labeling efficiency was checked by running the labeled, unlabeled and positive control on a 1% (w/v) agarose gel, where the labeled probe migrates slower than the unlabeled due to the presence of DIG. For the gel blot membranes, six µg total RNA was denatured in formamide with loading buffer and run on a 0.8% (w/v) Tris-Borate/EDTA (TBE) prepared agarose gel. RNA was transferred to a positively charged nylon membrane (Roche Diagnostics, Mannheim, Germany) by upward capillary blotting (Sambrook *et al.* 1989) using 10xSSC (standard saline citrate). RNA was UV cross-linked for 2.5 minutes at 120 mJ/cm using a UV cross-linker (Ultra Lum, (CA, USA)). All hybridisation and washing procedures were carried out as described by the DIG Application Manual for Filter Hybridisation (Roche Diagnostics, Mannheim, Germany).

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) assay

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity was measured according to Velasco *et al.* (1994) modified to microtiter plate format. For the protein extraction, 200 mg root material was resuspended in 800 µl extraction buffer (50 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 1 mM EDTA, 0.1% (v/v) β-mercaptoethanol, 20% (v/v) glycerol). The homogenate was cleared by centrifugation at 13 000 rpm for 15 minutes at 4°C and desalted on Sephadex G-25 (medium) (Sigma Aldrich, St. Louis, Missouri, USA) using the extraction buffer, omitting β-mercaptoethanol. The assay mixture contained 100 mM Tris-HCl (pH7.6), 10 mM $MgCl_2$, 0.1% (v/v) β-mercaptoethanol, 5 mM ATP, 0.2 mM NADH and 20 units phosphoglycerate kinase. GAPDH was assayed with 100 µl root extract. The reaction was started by adding 3-phosphoglycerate to 4 mM. GAPDH activity was determined by measuring the decrease in the concentration of NADH at 340 nm for 5 minutes at room temperature, using a Power $Wave_x$ microplate scanning spectrophotometer (Bio-Tek Instruments, Winooski, Vermont, USA).

Glutamine synthetase assay

Glutamine synthetase (GS) activity was measured as described in Debouba *et al.* (2006) and Wallsgrove *et al.* (1979). For the protein extraction, root material (200 mg) was resuspended in 800 µl extraction buffer (25 mM Tris-HCl (pH 7.6), 1 mM $MgCl_2$, 1 mM EDTA, 14 mM β-mercaptoethanol, 1% (w/v) PVP). The samples were incubated on ice for 15 minutes with intermitted vortexing and thereafter cell debris pelleted by centrifugation at 13 000 rpm for 30 minutes at 4°C to rendering a clear supernatant. For the assay, 600 µl root extract was used. Glutamine synthetase activity was determined using hydroxylamine as substrate, and the formation of γ – glutamylhydroxamate (γ – GHM) was quantified with acidified ferric chloride as described in Wallsgrove *et al.* (1979). The assay mixture (400 µl) contained 5 µmol aspartate, 45 µmol $MgCl_2$, 6 µmol hydroxylamine, 87 µmol glutamate and 45 µmol imidazole (pH 7.2). Samples were incubated at 30°C for 40 minutes and the reaction stopped by the addition of 1.0 ml $FeCl_3$ reagent (0.37 M $FeCl_3$, 0.67 M HCl, 0.2 M trichloroacetic acid). Thereafter the samples were cleared by centrifugation for 5 minutes as 1 000 x g. The absorbance of the supernatant was measured at 500 nm using a Power $Wave_x$ microplate scanning spectrophotometer (Bio-Tek Instruments, Winooski, Vermont, USA) and compared to that of an authentic γ – glutamyhydroxamate standard under the same conditions.

Western blot analysis

Proteins were extracted from approximately 200 mg of ground root material on ice for 15 min in 4 volumes of freshly prepared extraction buffer (25 mM Tris-HCl (pH 7.6), 1 mM $MgCl_2$, 1 mM EDTA, 14 mM β-mercaptoethanol, 1% (w/v) polyvinylpyrrolidone (PVP)). Cell debris was precipitated by centrifugation (30 min, 13000rpm, 4°C) to render a clear supernatant. Proteins were quantified using the Bradford method (Bradford, 1976) using the Bio-Rad reagent (Bio-Rad, Hercules, CA, USA) and BSA as a standard. For the western blot analysis, 25 microgram of protein per sample was precipitated with 4 volumes of 100% (v/v) acetone and incubated for 60 minutes at -20°C. Precipitated proteins were collected by centrifugation at 13 000 rpm for 10 minutes at 4°C. The protein pellet was resuspended in 20 µl sample buffer and heated for 5 minutes at 95°C, thereafter resolved on a 12% SDS-PAGE according to Laemmli (1970). Proteins were blotted on a Hybond-C (AEC-Amersham) nitrocellulose membrane using a semi-dry transfer unit (Transblot; BioRad, Hercules, CA) according to manufacturers' instructions. Protein transfer was verified by staining with 0.2 % (w/v) Ponceau-Solution for 5 minutes, washed and blocked overnight in 4% (w/v) BSA in TBST-buffer and then incubated for 4 h in 4% (w/v) BSA in TBST-buffer containing the primary antibody, which was a 1:4000 dilution of the GS antiserum. Thereafter the blot was rinsed

several times with TBST-buffer. The secondary antibody (1:2000 dilution of an alkaline-phosphatase-conjugated goat anti-rabbit IgG antibody (Sigma Aldrich, St. Louis, Missouri, USA) was added, the blot was washed again and developed through the enzymatic cleavage of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) using nitro blue tetrazolium (NBT) to give the precipitated indigo-derivate (NBT/BCIP, Roche Diagnostics, Mannheim, Germany).

RESULTS AND DISCUSSION

PLANT GROWTH STUDIES

Lumichrome promotes growth in Lotus japonicus at early growth stages and in different growth environments

Previous studies have reported that lumichrome promotes growth in different species at 5 nM (Phillips *et al.*, 1999; Matiru and Dakora, 2005a; Matiru and Dakora, 2005b; Khan *et al.*, 2008) and therefore this concentration was chosen. All plant growth experiments were undertaken in the absence of rhizobia in order to prevent interference with the supply of lumichrome. Firstly, growth experiments were conducted in the growth chamber. The seedling length was measured to asses the influence of lumichrome on the growth of seedlings. The lengths of 1 ½ week old seedlings were measured and were significantly ($P \leq 0.01$) longer compared to untreated control plants, indicating that growth promotion already occurs at early stages of growth and development (Figure 3.1 A). This was in agreement with Matiru and Dakora (2005a), where lumichrome was found to stimulate seedling development of certain legumes and cereals. Plant dry matter of five week old *Lotus japonicus* plants revealed that lumichrome promoted growth in *Lotus japonicus* roots, however significant ($P \leq 0.01$) differences were not present in the shoots (Figure 3.1 B). As a result, total plant biomass was increased, when *Lotus japonicus* was treated with 5 nM lumichrome. In addition, the flowers from plants treated with lumichrome appeared at an earlier stage as untreated control plants, thus inducing early flowering in *Lotus japonicus*. Chen *et al.* (2007) showed that foliar application of LCOs (lipo-chitooligosaccharide) enhanced flowering and fruit production. Secondly, growth experiments were conducted in tissue culture and showed that, compared to the untreated control plants, lumichrome significantly ($P \leq 0.01$) increased whole plant biomass in *Lotus* seedlings (Figure 3.2).

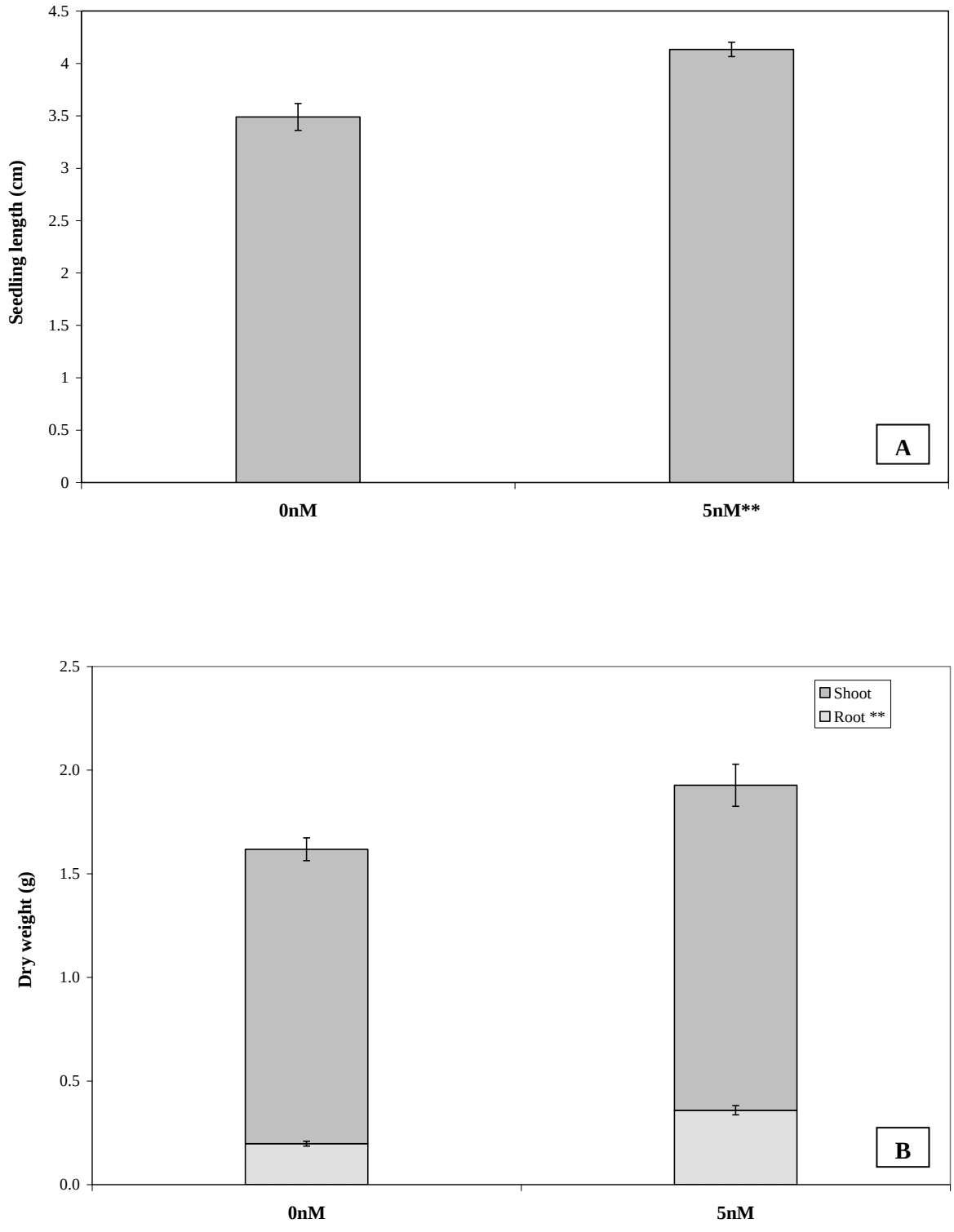


Figure 3.1 Lumichrome significantly promotes growth in early stages of development in *Lotus japonicus* seedlings (A) as well as in the roots (B) of five week old plants in the growth chamber. Mean values (± SE) are reported for nine (A) and five (B) independent replicates. ** Indicate treatment effects statistically significant at $P \leq 0.01$, determined by Student t-test.

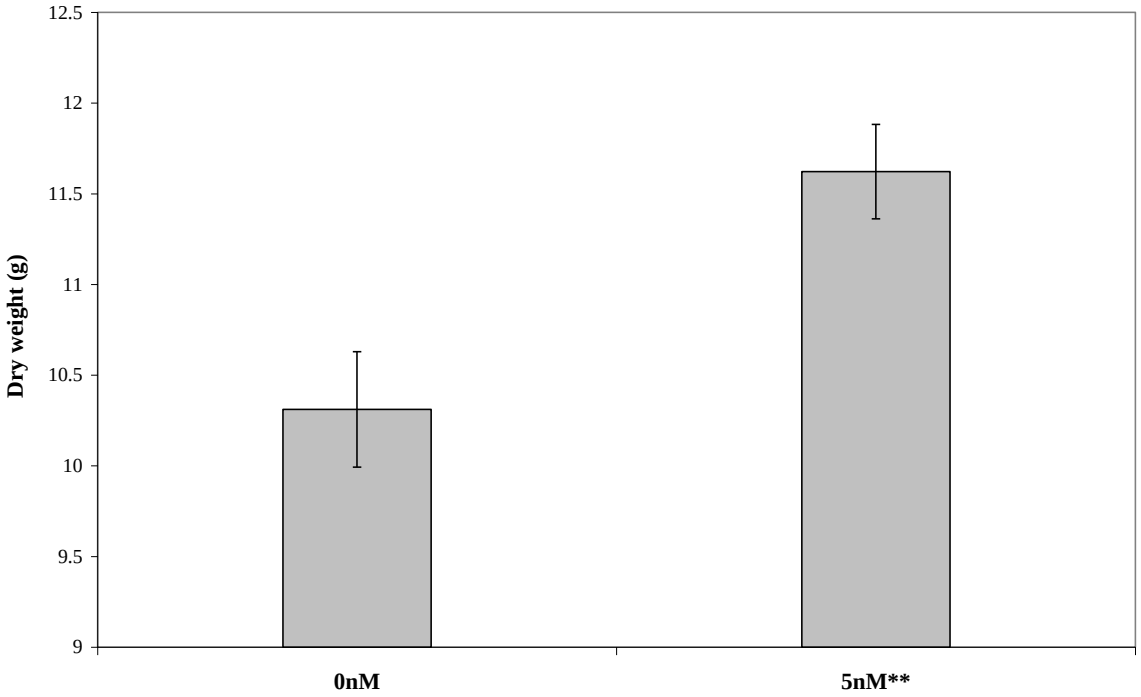


Figure 3.2 Lumichrome significantly increases whole plant biomass of *Lotus japonicus* in tissue culture. Mean values (± SE) are reported for nine independent replicates. ** Indicate treatment effects statistically significant at $P \leq 0.01$, determined by Student t-test.

TRANSCRIPT PROFILING

Transcript profiling of *Lotus japonicus* roots in response to lumichrome

To investigate the underlying mechanism of lumichrome mediated growth promotion, gene expression profiling on the mRNA level of *Lotus japonicus* lumichrome treated and untreated control roots were performed. DNA microarrays were utilized to reveal genes that were differentially expressed. The application of lumichrome led to statistically significant changes in 168 transcripts, where 40% of the genes were induced and 4% repressed in response to lumichrome treatment (Figure 3.3 B). Out of the 168 genes, 56% of the genes were not included as the sequences displayed no similarity to known proteins or were homologous to hypothetical proteins. The complete data set of significantly altered transcripts is available in the supplementary data section (Table A and B). An overview of gene expression categorized in functional groups provides additional information as to which biological process was most affected by lumichrome. Elements that were differentially expressed were categorically divided into cell division and differentiation, protein interaction, modification and folding,

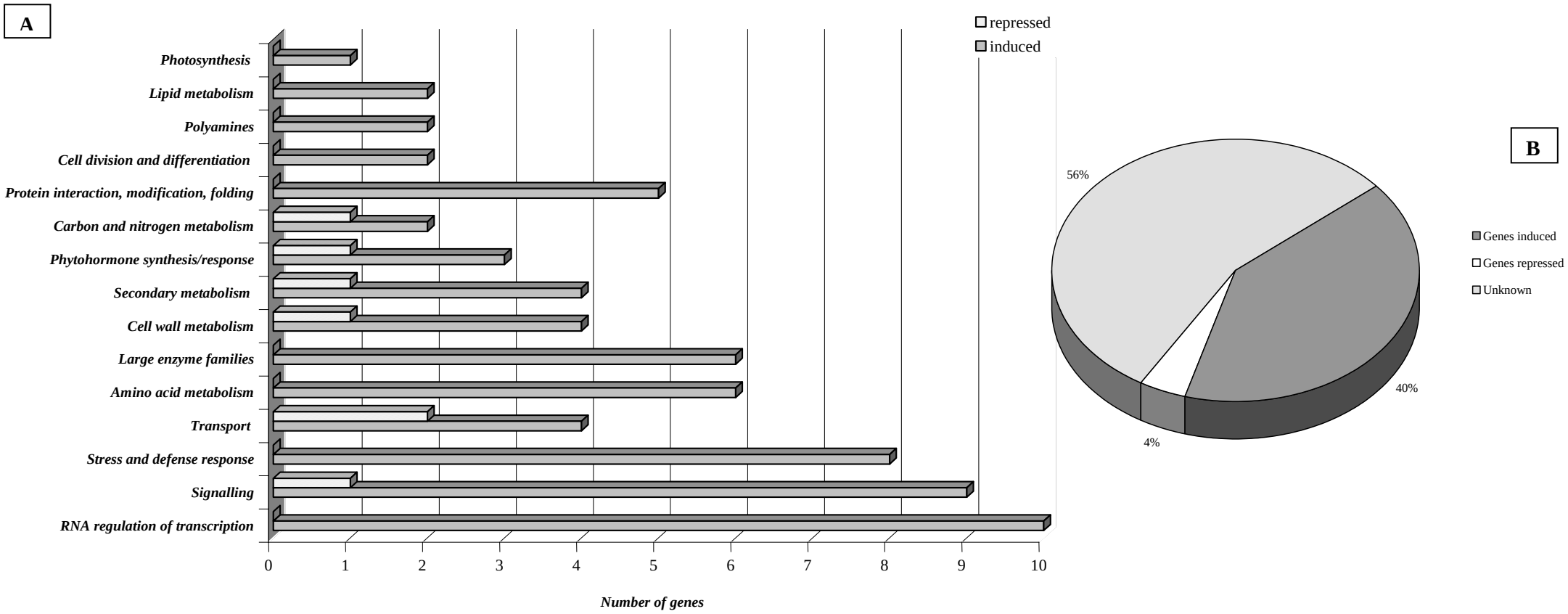


Figure 3.3 Differential gene expression of *Lotus japonicus* roots as a result of lumichrome treatment.

A) Overview of induced and repressed biological processes in treated roots. The number of genes found in each category is identified on the *x*-axis. Genes of unknown function or classification are not shown. **B)** The pie chart shows the percentage (%) of genes induced, repressed and unknown genes that could not be identified as they displayed no similarity to known proteins or were homologous to hypothetical proteins.

RNA regulation of transcription, carbon and nitrogen metabolism, phytohormone synthesis and response, secondary metabolism, cell wall metabolism, large enzyme families, amino acid metabolism, transport, stress and defence and signaling, photosynthesis, lipid metabolism and polyamines (Figure 3.3 A). RNA regulation of transcription, signaling and stress and defence were the top three categories that showed the most changes regarding differential gene expression, with the RNA regulation of transcription and signaling categories displaying the highest number of changes in gene expression.

Signaling and RNA regulation of transcription

The observed changes in gene expression regarding signaling events and RNA regulation of transcription were immense. In plants, transcription factors are important regulatory switches that control many aspects of plant development and transcription factors are final mediators of most signaling cascades that lead to specific protein/DNA interactions and modulation of gene expression (Shepard and Purugganan, 2002; Vargas *et al.*, 2003). The gene annotated to a lateral organ boundaries domain 30 (LBD30), which constitute a novel class of DNA-binding transcription factors (Husbands *et al.*, 2007), was up-regulated in lumichrome treated plants. The *Arabidopsis LATERAL ORGAN BOUNDARIES (LOB)* gene is expressed at the boundaries of lateral organs during vegetative and reproductive plant development. The LOB gene encodes a protein containing a concerved amino acid domain, termed the LOB domain (LBD) and LBD genes are only found in plants (Shuai *et al.*, 2002). The orthologous *Arabidopsis* LBD genes are needed for lateral root formation and directly regulated by auxin response factors (ARF) (Okushima *et al.*, 2005; Okushima *et al.*, 2007). Thus, LBD genes are important regulators of developmental processes. Another gene that was highly induced was a homeodomain transcription factor BLH1. Hoth *et al.* (2002) investigated abscisic acid (ABA)-responsive gene expression and this homeodomain transcription factor was up-regulated upon ABA treatment, thus the up-regulation of this transcription factor in lumichrome treated roots might be in response to the phytohormone ABA. Husbands *et al.* (2007) demonstrated that members of the basic-helix-loop-helix (bHLH) family of transcription factors, which was also up-regulated in treated roots, are capable of interacting with LOB. Furthermore, the gene expression levels of the gene annotated to the polyphosphoinositide binding protein (SSH2P) was up-regulated. This protein is part of the phosphatidylinositol transfer protein (PITPs) and modulates signal transduction pathways and membrane trafficking (Kapranov *et al.*, 2001). The gene annotated to a MYB68 transcription factor was up-regulated. The MYB proteins are a superfamily of transcription factors that play regulatory roles in developmental and defence processes and are usually responsive to

hormone and stress treatments. Specifically, MYB68 seem to respond to gibberellic acid (GA) and salicyclic acid (SA) treatments (Yanhui *et al.*, 2006). The gene annotated to the WRKY6 transcription factor was up-regulated. The expression of WRKY6 is often influenced by several external and internal stimuli often associated with senescence and plant defence. Defence response induced gene expression of the MYB68 and WRKY6 transcription factors and the up-regulation of a salt tolerance zinc finger, induced by dehydration and abscisic acid treatment (Sakamoto *et al.*, 2004), might indicate that the plant is perceiving lumichrome as a signal molecule for abiotic and biotic stress. The gene annotated to a specific MADS-box protein, *SHORT VEGETATIVE PHASE (SVP),* was highly induced upon lumichrome treatment. This gene mediates ambient temperature signaling in *Arabidopsis* and Lee *et al.* (2007) showed that the SVP-mediated control of *FLOWERING LOCUS T (FT)* expression is one of the molecular mechanisms developed by plants to modulate the timing of the developmental transition to the flowering phase, in response to changes in the ambient temperature. It is important to note that lumichrome induced early flowering in *Lotus japonicus*, therefore changes in the expression levels of this gene might have played an important role in reducing the flowering time. The gene annotated to histone deacetylase 2C (HD2C) was up-regulated and has been implicated to play a role specifically in the expression of patterning genes in the *Arabidopsis* root epidermis (Xu *et al.*, 2005), in regulating the gene expression of pathogenesis related genes and in promoting resistance to fungal pathogens (Pfluger and Wagner, 2007). Another interesting study revealed that in *Arabidopsis* HD2C is involved in ABA mediated abiotic stress responses (Sridha and Wu, 2006). The vast majority of the genes that were differentially expressed in the signaling category were protein kinases or receptor kinases. The gene annotated to MAPK3 was induced, which is involved in stress and pathogen signal transduction. Kovtun *et al.* (2000) could prove that several stress signals but specifically H_2O_2 can activate MAPK3. Furthermore, Lu *et al.* (2002) showed that apart from being activated by H_2O_2, MAPK3 is activated by ABA as well. Therefore, the aforementioned changes in gene expression points to the involvement of H_2O_2 and ABA in the lumichrome mediated growth promotion of *Lotus* roots as well as the induction of abiotic and biotic stress responses.

Stress and defence response

Three genes annotated to disease resistance were highly up-regulated at the mRNA level. Gene expression levels of the extracellular dermal glycoprotein (EDGP) were up-regulated, implicating an increase in biotic and abiotic stress. EDGP has been suggested to play a protective role in plant cell walls, as it inhibits the activity of xyloglucan-specific β-1,4-

endoglucanase from the fungus *Aspergillus aculeatus* (Shang *et al.*, 2004). Gene expression levels of alcohol dehydrogenase were up-regulated, which is induced by oxygen deficiency and shows mainly root specific expression (Dolferus *et al.*, 1997). Hairpin-induced proteins were induced upon lumichrome treatment. These proteins are produced by plants in reaction to hairpins, which are proteins produced by several Gram-negative plant pathogenic bacteria and activate numerous plant defence pathways in plants. A gene annotated to a NIMIN3 was up-regulated in response to lumichrome treated roots. NIMIN-1, NIMIN-2, NIMIN-3 (NIMIN for NIM-interacting) are members of a family of proteins from *Arabidopsis* that interact with NPR1/NIM1, which is a key regulator of systemic acquired resistance in plants (Weigel *et al.*, 2001). Emerging evidence shows that NIMINs act as negative regulators of NPR1 in modulating the expression of SA-inducible pathogenesis related (PR) proteins (Potlakayala *et al.*, 2007). The results indicate once more that the plant perceives lumichrome as a signal for biotic stress. In addition, the synthesis of phytoalexins seems to be induced through the up-regulation of N-hydroxycinnamoyl/benzoyltransferase.

Cell wall metabolism

Of great interest were the changes in gene expression in cell wall metabolism at the mRNA level. Changes in gene expression point to increases in cellulose synthesis, lignification and perhaps a mimicked response to pathogens or wounding. The transcript levels for cellulose synthase were highly up-regulated, which points to increased synthesis of the β-1,4-linked glucose polymer cellulose from UDP-glucose which is essential for cell wall formation. Moreover, laccases were up-regulated which might indicate an increase in lignification in lumichrome treated roots. In addition, there seems to be decreased cell elongation with the down-regulation of the mRNA for the enzyme β-1,4-glucanase, the enzyme thought to play an important role in elongation processes where it hydrolyzes a number of β-1,4-linked glucans and likely targets the glucan backbone of xyloglucan (Shani *et al.*, 2006) .

Secondary metabolism

Transcripts for cycloartenol synthase were up-regulated in lumichrome treated roots. Sterol biosynthesis is initiated by this specific enzyme. Interestingly, sterols are precursors of the phytohormones brassinosteroids, which might play a role in lumichrome mediated growth promotion. With increases in mRNA expression levels for chalcone synthase (CHS), which is the first committed enzyme in flavonoid synthesis and produces an intermediate used in the synthesis of all the flavonoids (Lillo *et al.*, 2008), flavonoid biosynthesis seems to increase upon lumichrome treatment. The fact that mRNAs for flavonoid synthesis are up-regulated in

lumichrome treated roots would make sense, as plants release flavonoid compounds that act as signals for bacteria. Lumichrome stems from rhizobacteria and it might be that it stimulates the production of flavonoids to attract more rhizobia that in turn, secrete Nod factors that increase plant growth. Furthermore, a gene annotated to anthocyanin acyltransferase was down-regulated, which is involved in flavonoid biosynthesis. Increased levels of gene expression were observed for phenylalanine ammonia lyase (PAL1), which provides precursors for lignin and phenols as well as for SA. This might point to increases in lignin synthesis, corroborating the changes that were observed in the cell wall metabolism.

Amino acid metabolism

The genes responsible for amino acid activation were highly up-regulated, specifically the genes annotated to phenylalanine-tRNA synthetase and aspartate-tRNA ligase. Phenylalanine is a vital amino acid in secondary metabolism and increases in gene expression of enzymes involved in secondary metabolism was observed in lumichrome treated roots in this study. The gene for phospho-2-dehydro-3-deoxyheptonate aldolase synthase (DAHP synthase), which catalyses the first reaction in the shikimate pathway, was induced in treated roots. A gene annotated to γ-aminobutyric acid (GABA) permease, an amino acid carrier, was highly up-regulated. GABA is synthesized by decarboxylation of glutamate and degraded via succinic semialdehyde to succinate, a pathway that is also called the GABA shunt. GABA has been discussed to be important for the regulation of cytosolic pH, nitrogen storage and metabolism and protection against oxidative stress (Shelp *et al.*, 1999; Bouche and Fromm, 2004). Moreover, it has been suggested that GABA plays a role in intercellular signaling in plants and is involved in communication between plants and bacteria (Shelp *et al.*, 2006).

Carbon and nitrogen metabolism

Sucrose synthase, which converts sucrose and UDP into UDP-Glucose and fructose (Tsai, 1974), is usually highly expressed in tap roots (Hardin *et al.*, 2006). However, in this study it was found that the gene expression levels for this enzyme was down-regulated compared to the untreated control roots. Plasma membrane associated sucrose synthase is postulated to channel its product, UDP-glucose, towards the synthesis of cellulose (Hardin *et al.*, 2006). As mentioned previously, cellulose synthase was up-regulated – therefore the UDP-glucose probably stems from another source, as the gene for sucrose synthase is down-regulated. A slight increase of glutamine synthetase gene expression levels was observed in treated roots. Glutamine synthetase catalyzes the incorporation of ammonia into glutamate to generate glutamine with concomitant hydrolysis of ATP and plays a crucial role in the assimilation and

re-assimilation of ammonia derived from a wide variety of metabolic processes during plant growth and development (Unno *et al.*, 2006). Interestingly, trehalase was up-regulated in treated plants. Trehalase catalysis the hydrolysis of trehalose (α-D-glucopyranosyl-1,1-α-D-glucopyranoside) and plant trehalase activity and transcripts seem to be induced by biotic and abiotic stress and acts as a sensor and regulator of carbon allocation (Frisona *et al.*, 2007; Müller *et al.*, 2001). Furthermore, trehalase activity has been found to be increased in symbiotic relationships of plants with microorganisms such as rhizobia (Müller *et al.*, 1994). Lumichrome is a metabolite stemming from rhizobia, which might be a possible reason for the increase in gene expression at the mRNA level.

Large enzyme families

Transcript levels for peroxidases were increased in lumichrome treated plants. Peroxidases utilize either H_2O_2 to oxidize various phenolic substrates producing polymeric products such as lignin to crosslink cell wall polymers, leading to stiffening and inhibition of elongation (Maksimović *et al.*, 2008). Maksimović *et al.* (2008) showed that increases in peroxidase activities were paralleled by the increase of coniferyl alcohol and other phenolic compound concentrations with root and leaf age, indicating that increased concentrations reflected increased lignification during maturation. Therefore, it is likely that the peroxidases play a vital role in the lumichrome mediated growth promotion of the *Lotus* roots. The gene expression levels for cytochrome P450 were up-regulated at the mRNA level. Cytochrome P450 monooxygenase is a heme-containing enzyme family that catalyses the oxidative reaction of a wide variety of organic compounds by utilizing atmospheric O_2. In higher plants P450s are also involved in the biosynthesis of fatty acids, GAs and a variety of secondary metabolites such as phenylpropanoids, terpenoids and sterols (Donaldson and Luster, 1991; Bollwell *et al.*, 1994). Moreover, P starvation and NO induced gene expression of cytochrome P450 monooxygenases (Valdés-López and Hernández, 2008; Grün *et al.*, 2006). The gene for glutathione-S-transferase (GST) was up-regulated in lumichrome treated plants, which plays a role in both normal cellular metabolism as well as in the detoxification of a wide variety of xenobiotic compounds and oxidative stress responses. GSTs can be implicated in various stress responses, including those arising from pathogen attack, oxidative stress and heavy metal toxicity and GSTs play a role in the cellular response to auxins and during the normal metabolism of plant secondary products like anthocyanins and cinnamic acid. It might be that the plant senses lumichrome as a toxic compound, therefore implementing the common pathway that exists in most organisms for the detoxification of electrophilic compounds with enzymes such as cytochrome P450 monooxygenases, UDP:glucosyltransferases and ATP-

dependant membrane pumps (Marrs, 1996). Also the genes for glucosyltransferases, which catalyse the transfer of sugars to a wide range of acceptor molecules, were highly up-regulated at the mRNA level.

Phytohormone synthesis and response

Regarding phytohormone synthesis and response, only genes encoding enzymes which are involved in ethylene and cytokinin synthesis showed differential expression. Ethylene levels seem to be elevated due to the increase in transcripts of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) which is the rate-limiting step in ethylene biosynthesis. Additionally, the ethylene-regulated transcript 2 (ERT2) was up-regulated. It is known to code for an ethylene-regulated nuclear protein ERN1 and it has been suggested to play a role in plant growth and flower development, which are both processes known to be affected by ethylene (Trentmann, 2000). A vast majority of genes pointed to changes pointed to changes in ABA synthesis.

Protein interaction, modification and folding

Some genes encoding proteins falling into the category protein interaction, modification and folding were up-regulated. Increased transcript levels for heat shock proteins and co-chaperones were observed. In response to abiotic stresses, these proteins are vital as they assist in protein folding and prevent protein denaturation. A gene annotated to a CP12 precursor was up-regulated. CP12 was originally identified as a small, redox-sensitive, chloroplast protein that interacts with two enzymes of the Calvin cycle, phosphoribulokinase (PRK) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forming a high molecular weight complex (PRK/GAPDH/CP12) (Wedel *et al.*, 1997; Graciet *et al.*, 2003). However, recent work by Singh *et al.* (2008) showed the expression of CP12 on the tip and newly emerging lateral roots, indicating a role for the CP12 in meristematic tissue and cells undergoing differentiation. Therefore, one might postulate the involvement of CP12 in the increase in lateral roots and root biomass. GrpE proteins are one of the co-chaperones of the Heat shock protein 70 (Hsp70), which are essential components of protein metabolism both in the cytosol and in organelles. They play active roles in regulating cellular events such as protein degradation, morphogenesis and cell cycle progression (Cagliari *et al.*, 2005; Hartl, 1996). Transcripts for GrpE proteins were up-regulated in lumichrome treated plants. Another interesting gene that was induced by the treatment of lumichrome, was the gene annotated to a auxilin-like protein. Auxilin has been shown to aid in the uncoating clathrin–coated vesicles vesicles through the interaction with the Heat shock protein 70 (Lam *et al.*, 2001).

Lipid metabolism

Genes for lipases were up-regulated in lumichrome treated plants at the mRNA level. Lipases play a role in lipid metabolism where they catalyze the hydrolysis of fatty acids from the glycerol backbone. Specifically, the gene expression levels of GDSL-motif lipase/acylhydrolases were up-regulated. This motif defines a large family of characterized lipolytic enzymes. Naranjo *et al.* (2006) showed that the overexpression of *AtLTL1*, which encodes a lipase of the GDSL-motif family, increased salt tolerance and vegetative growth in transgenic plants and moreover, salicylic acid (SA) activated its expression suggesting that the lipase could be involved in defence reactions against pathogens. Generally, defence reactions against pathogens seem to be induced in lumichrome treated plants. Gene expression levels of acetoacetyl-CoA thiolase were highly up-regulated in lumichrome treated roots. acetoacetyl-CoA thiolase catalyses the conversion of acetyl-CoA into acetoacetyl-CoA, which is the first step in the biosynthesis of steroidal backbones. Sterols, which are biosynthesized via the cytoplasmic mevalonate (MVA) pathway, are important structural components of the plasma membrane and precursors of steroid hormones in plants, which might point to the synthesis and importance of brassinosteroids in promoting plant growth (Benveniste, 2004; Schaller, 2004; Müssig, 2005). It seems that this pathway is up-regulated in lumichrome treated roots.

Transport

Genes for transport processes displayed marked changes in gene expression at the mRNA level. There seem to be a strong increase in vesicle trafficking in treated plants, as the gene for Snap25a was highly induced. The SNAP25-type proteins belong to the superfamily of the SNAREs (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors), and function as important components of the vesical trafficking machinery in eukaryotic cells. As the roots displayed increases in growth, increases in cell division is a likely explanation for the induction. Diverse cell components must be transported in order to keep up with the demands of cell division. Lumichrome stems from the rhizobacteria *Sinorhizobium meliloti*, therefore the expression of Snap25a might also be a reaction to pathogen infection, given that the Snap-25 family is induced upon pathogen infection and biotic and abiotic stresses (Bao *et al.*, 2008; Hiraoka *et al.*, 2009). Additionally, a gene annotated to encode a plasma membrane intrinsic protein, belonging to the family of aquaporins, was induced. Aquaporins are channel proteins that transport water and certain neutral metabolites across biological membranes (Chaumont *et al.*, 2001). The up-regulation of transcripts for aquaporins might point to increased water transport into the cytoplasm and vacuole, consequently altering turgor

pressure and ultimately contribute to cell expansion and growth. Interestingly, the plasma membrane Ca^{2+}-ATPase was down-regulated which might also point to the fact that the plant is perceiving lumichrome as a stress signal as this ATPase is a major Ca^{2+} regulator in stress situations where the cytosolic Ca^{2+} has been shifted from its steady-state level (Felle *et al.*, 1992).

Polyamines

Polyamines contribute to various processes such as root development (Couée *et al.*, 2004), differentiation and development of flowers and fruits (Antognoni *et al.*, 2002) and cell wall loosening and stiffening (Cona *et al.*, 2003). The gene annotated to encode ornithine decarboxylase was up-regulated in lumichrome treated roots. In plants, the diamine putrescine is synthesized directly from ornithine by ornithine decarboxylase (Delis *et al.*, 2005) and is an essential primary metabolite for normal growth and development. Another gene encoding an enzyme involved in polyamine metabolism, amine oxidase was up-regulated. Amine oxidase catalyzes the oxidative de-amination of polyamines, thus degrades cellular polyamines and contributes to polyamine homeostasis. The production of hydrogen peroxide (H_2O_2) deriving from polyamine oxidation has been correlated with cell wall maturation and lignification, wound healing and cell wall reinforcement during pathogen invasion. In turn, H_2O_2 mediates hypersensitive response and the expression of defence genes (Cona *et al.*, 2006). In particular, 4-aminobutanal, which is another product of polyamine oxidation, can be further metabolized to GABA (Bouchereau *et al.*, 1999). Other products of polyamine oxidation are involved in secondary metabolite synthesis and abiotic stress tolerance (Cona *et al.*, 2006). The levels of polyamines change under stress conditions such as osmotic stress, mineral deficiencies, chilling, wounding and salinity and is used as a stress indicator (Delis *et al.*, 2005). In specific tissues in *Arabidopsis* seedlings, polyamines induce NO biosynthesis, especially in the elongation zone of root tips and primary leaves (Tun *et al.*, 2006). These observations are all valid points to examine as lumichrome seems to induce various responses for example the increased production of secondary metabolites, exhibit signs of phosphate and nitrogen deficiencies as well as increased root growth and lastly, induces typical pathogen responses.

PROTEIN PROFILING

Proteomic analysis of lumichrome-induced changes in *Lotus japonicus* roots

To investigate key proteins that were differentially expressed as a result of lumichrome treatment and play a pivotal role in growth stimulation, proteins were extracted from *Lotus*

japonicus roots and analyzed with 2-D gel electrophoresis. Comparing the 2-DE spot patterns of untreated control samples (Figure 3.4 A) and treated samples (Figure 3.4 B) with the Melanie 2D – Gel Evaluation Software resulted in the detection of 233 differentially expressed proteins. 15 Selected spots were manually excised, digested with trypsin and analysed by MALDI-MS or nano-LC-ESI-MS/MS. Among the 15 spots that were analyzed, 5 spots could be identified (3 spots = Nano-LC-ESI-MS/MS; 2 spots = MALDI-MS) (Table 1).

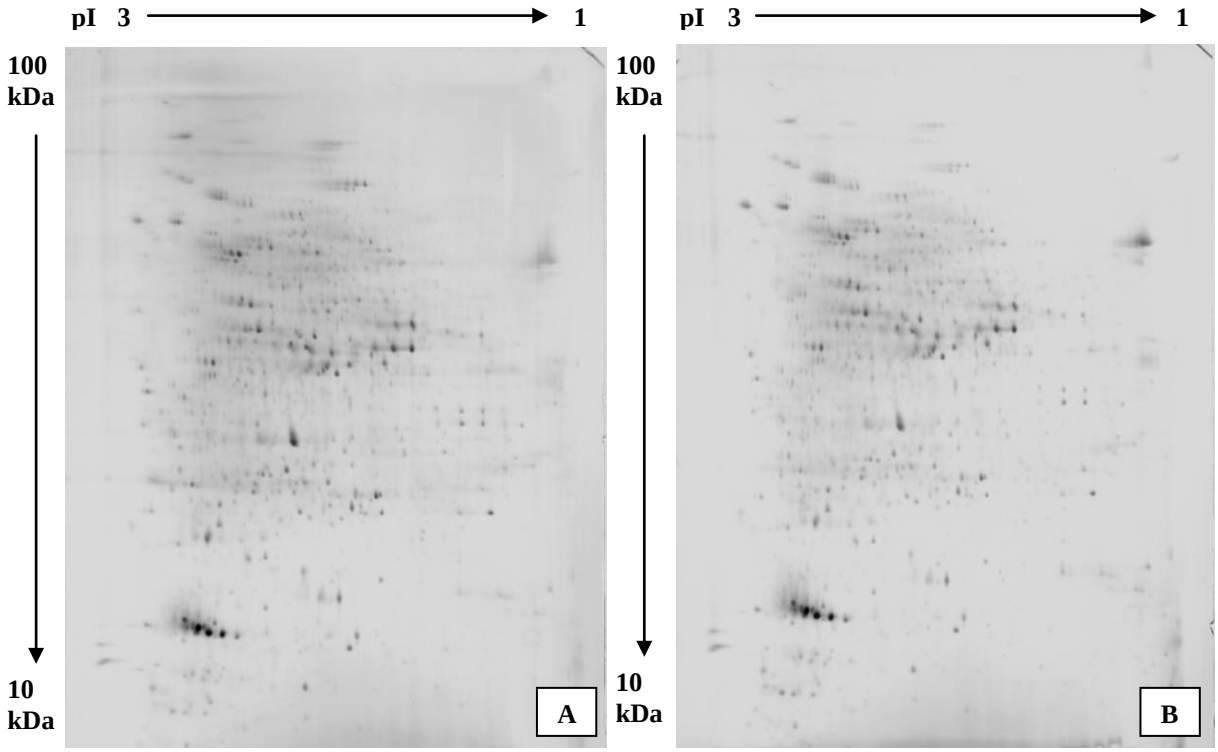


Figure 3.4 2-D gel analysis of *Lotus japonicus* roots performed with the NEPGHE system. Proteins were extracted from control plants (A) and lumichrome treated (B) plants. 400 µg of total protein was loaded for the IEF in the first dimension.

Spot 174 (Figure 3.5) was identified as the Heat shock protein 70 (HSP70), which was down-regulated. HSP70 interacts with other chaperones and exchange unfolded polypeptides with them and their involvement includes protein folding, unfolding, assembly and disassembly. Members of the Hsp70 family are expressed in response to environmental or abiotic stress conditions (Cagliari *et al.*, 2005). *Spot 675* (Figure 3.5) was identified as cytosolic glutamine synthetase (GS1). GS is the key enzyme involved in ammonia assimilation in plants and catalyzes the ATP-dependant condensation of NH_3 with glutamate to produce glutamine. In plants GS is located either in the cytoplasm (GS1) or in the chloroplast (GS2) (Temple *et al* 1998). GS1 was down-regulated in lumichrome treated roots. However, the microarray analysis showed increased transcript levels of GS1. Possible explanations together with

results of more targeted analysis are discussed in the following section: Targeted analysis of GS1 and GAPDH. Furthermore, the cytosolic glyceraldehyde-3-phosphate dehydrogenase (phosphorylating NAD-specific) (*Spot 810*; Figure 3.5) was differentially expressed and was down-regulated in lumichrome treated roots. GAPDH is a key enzyme in glycolysis and catalyses the conversion of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate (Velasco *et al* 1994). Proton-pumping ATPases, or F-type ATPases, are found in the inner mitochondrial and thylakoid membranes that synthesize ATP. The F-type ATPase comprises of two sectors, F_0 and F_1. The common subunits of F_0 are called a, b, c and F_1 comprises of five subunits called $\alpha_3\beta_3\gamma\delta\varepsilon$. With the 2-DE analysis, lumichrome treated roots showed a reduction in protein levels of the α-subunit compared to the control (*Spot 365*; Figure 3.6). *Spot 1482* (Figure 3.6) was identified as a Fe-superoxide dismutase precursor-like protein which was down-regulated. Superoxide dismutases are metalloenzymes that catalyze the dismutation of superoxide radicles to molecular oxygen and H_2O_2 (McCord and Fridovich, 1969).

Table 1 Identification of significantly differentially expressed proteins in response to lumichrome in *Lotus* roots. Spot numbers correspond to those indicated in Figure 3.5 and Figure 3.6

GROUP ID	***MW* (KDA)***	***CHANGE***	***PROTEIN NAME***	***MOWSE SCORE*******	***MEANS OF ID***
174	81	0.67***	HSP 70 (Medicago truncatula)	72/59	Nano-LC-ESI-MS/MS
365	61	0.56**	F1 ATPase subunit alpha	106/68	MALDI-MS
675	44	0.63***	Glutamine synthetase cytosolic isoenzyme	384/59	Nano-LC-ESI-MS/MS
810	39	0.56***	Glyceraldehyde-3-phosphate dehydrogenase	67/59	Nano-LC-ESI-MS/MS
1482	18	0.63**	Fe-superoxide dismutase precursor-like	80/68	MALDI-MS

* Molecular mass of the protein match ** significance level at < 0.05 *** significance level at <0.001

**** MOWSE = molecular weight search, MOWSE score – statistical probability of true positive identification of predicted proteins.

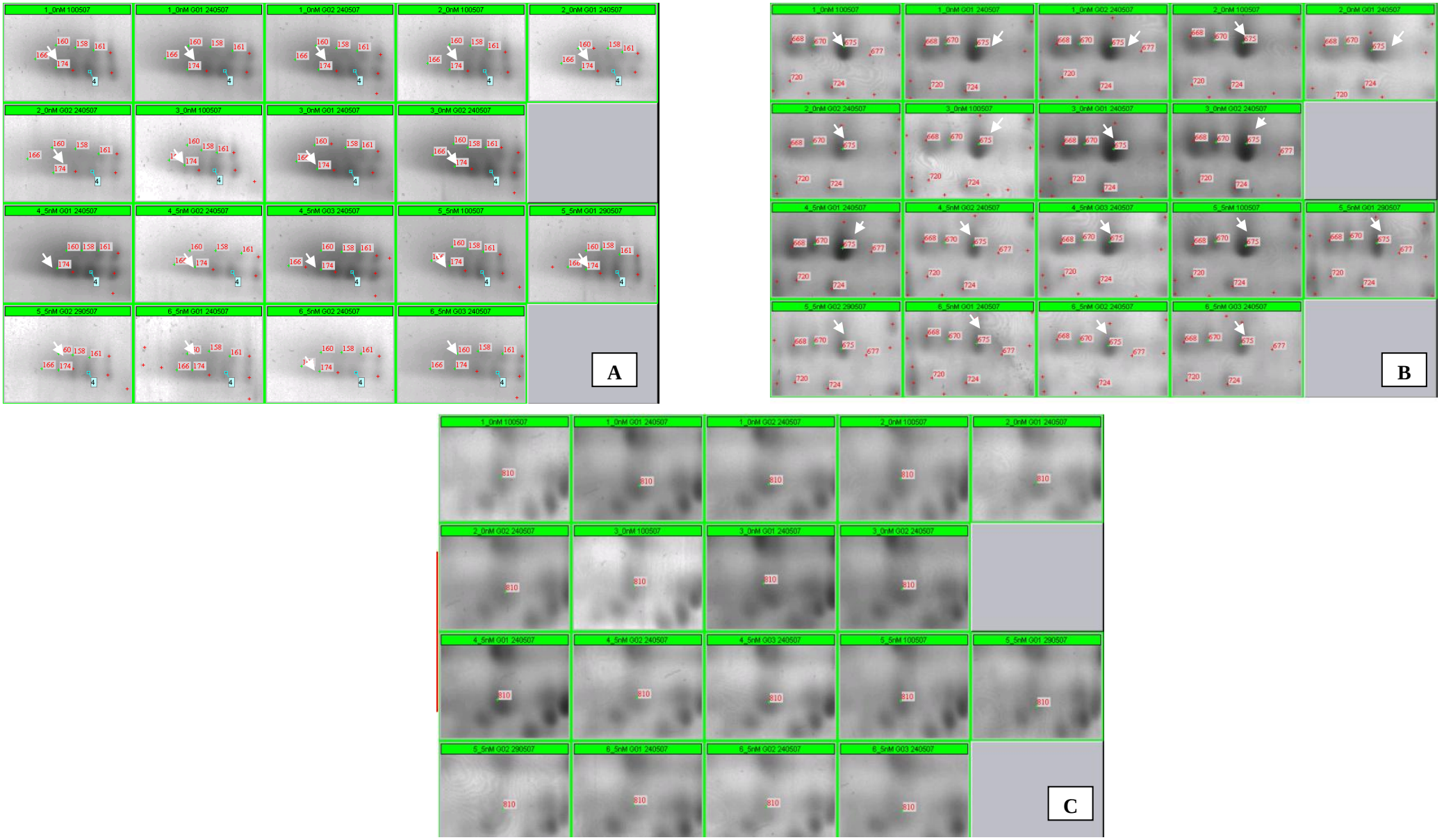
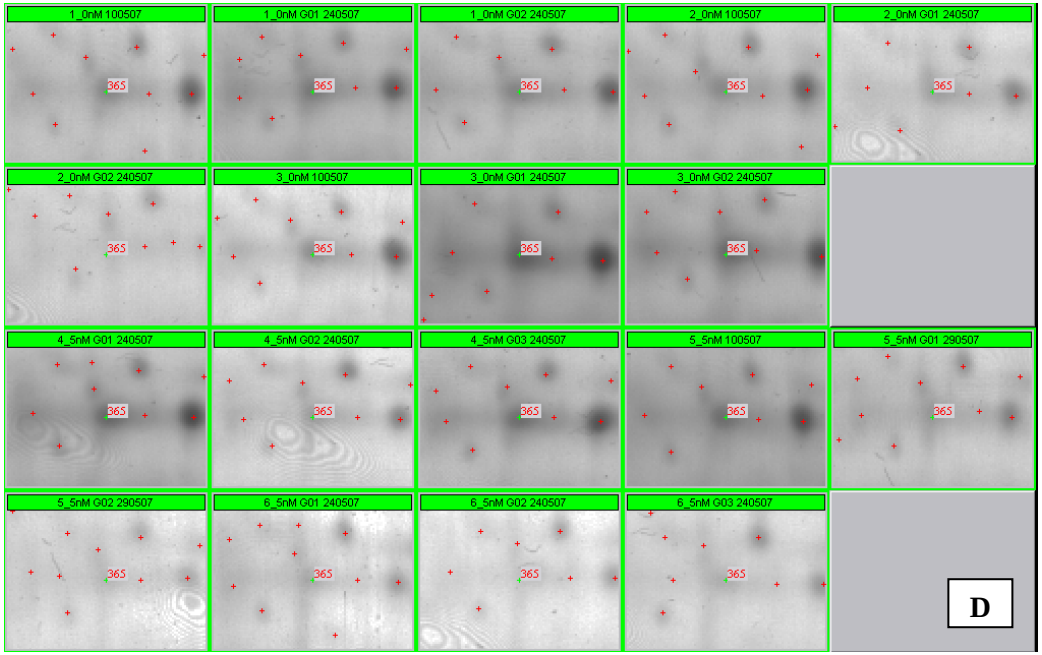


Figure 3.5 Comparison of protein spots originating from 2-D gels of lumichrome treated and untreated *Lotus japonicus* roots. (A) Spot 174 = HSP 70; (B) Spot 675 = Glutamine synthetase 1; (C) Spot 810 = Glyceraldehyde-3-phosphate dehydrogenase. In A, B and C the first two rows represent untreated control samples (sample 1-3) and the bottom two rows lumichrome treated samples (4-6). All samples were run in triplicate and all three proteins were down-regulated. For grouping of samples, see Table 1.



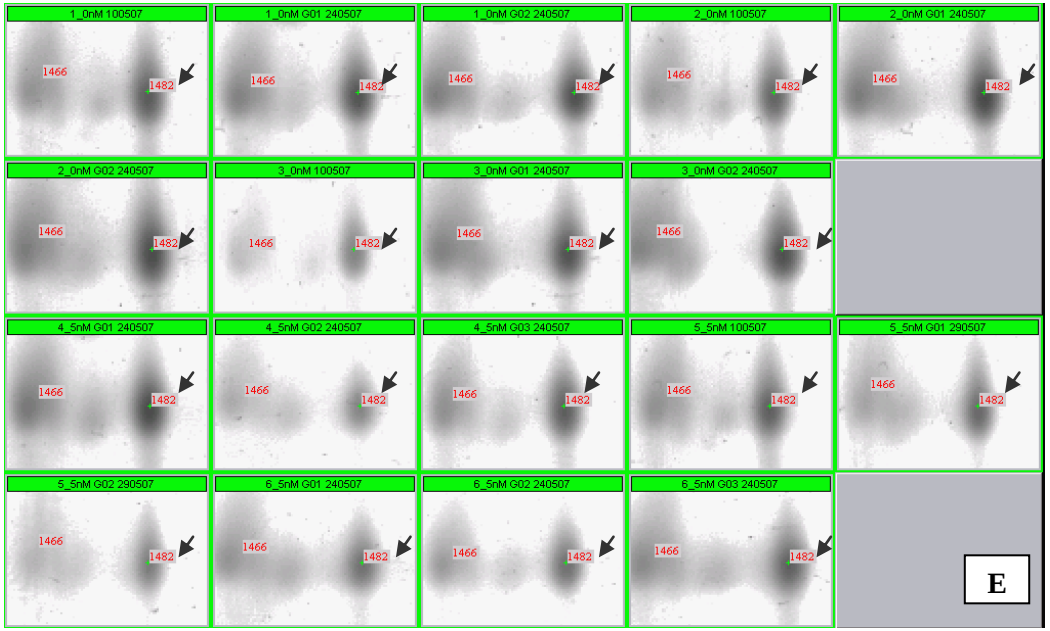


Figure 3.6 Comparison of protein spots originating from 2-D gels of lumichrome treated and untreated *Lotus japonicus* roots. (D) Spot 365 = F1 ATPase; (E) Spot 1482 = Fe-superoxide dismutase precursor-like. In D and E, the first two rows represent untreated control samples (sample 1-3) and the bottom two rows lumichrome treated samples (4-6). All samples were run in triplicate and both proteins were down-regulated. For grouping of samples, see Table 1.

METABOLITE PROFILING

The influence of lumichrome on the metabolite levels of *Lotus japonicus* roots point to nitrogen deficiency and oxidative stress

As a first study aimed at understanding the influence of lumichrome on the metabolite composition of *Lotus japonicus*, metabolite profiling was utilized to gain insight into the response of *Lotus japonicus* roots. A total of 78 metabolites were measured and significant differences between samples were assessed by the Student *t*-test ($P \leq 0.05$). From the 78 metabolites that were measured, 46 metabolites were significantly changed in their abundance, indicating a widespread perturbation of metabolism in response to lumichrome treatment (Supplementary data, Table C). Usually, metabolite analysis is accompanied by large sets of data. It can become quite difficult to integrate different data sets and retrieve valuable information from it. To compare samples in the right manner, one needs to make use of a multivariate statistical tool such as principal component analysis (PCA) (Jonsson *et al.*, 2005).

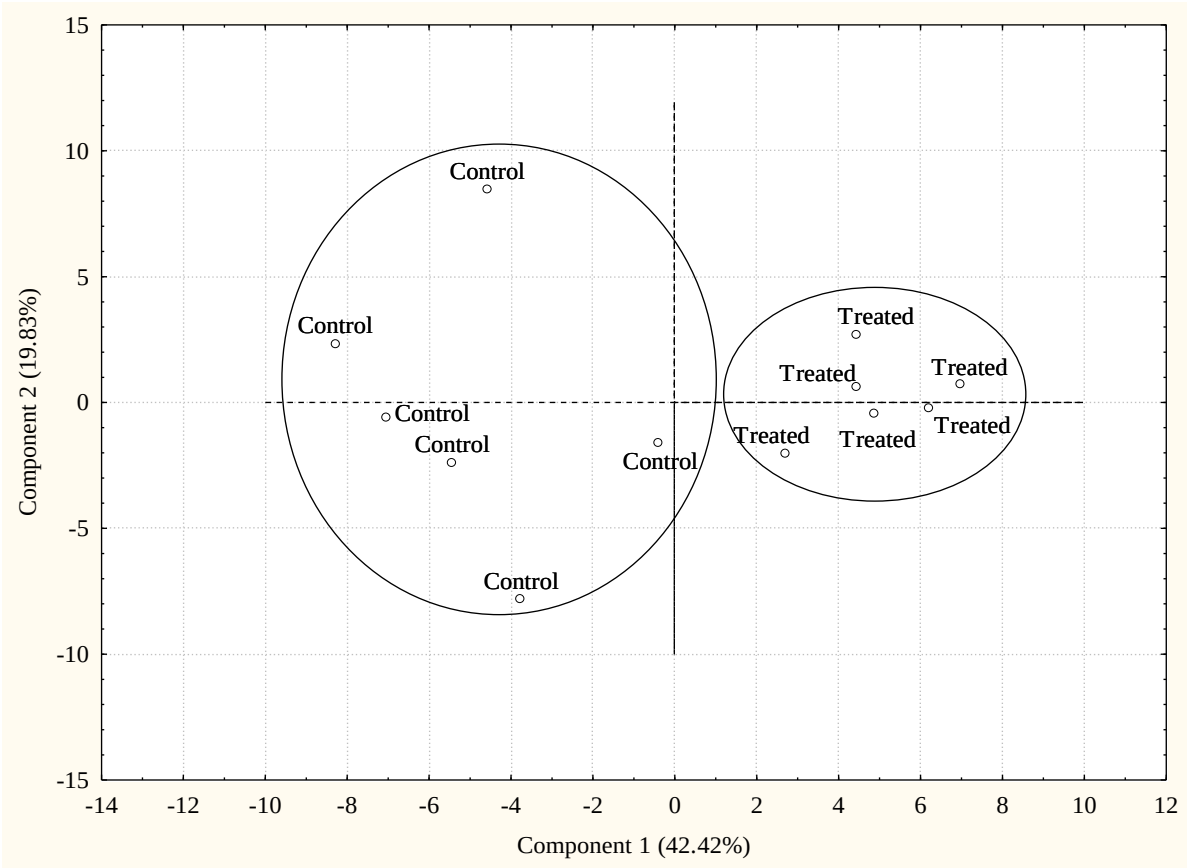


Figure 3.7 Principal Component Analysis (PCA) of 78 measured metabolites of *Lotus japonicus* roots, showing distinct groupings of lumichrome treated (n = 6) and untreated control (n = 6) plants.

PCA describes the largest variation in data using a few orthogonal latent variables. An overview of the data is obtained whereby trends, groupings, and outliers can be detected. Thus, PCA is a way of identifying patterns in data and expressing them in such a way that similarities and differences are highlighted (Smith, 2002). Therefore, in order to bring to light differences and similarities of metabolite data obtained from lumichrome treated and untreated *Lotus japonicus* roots, a PCA with Statistica 8 © Statsoft was performed. Taking into account all 78 metabolites, the PCA analysis revealed that the metabolomes of lumichrome treated and untreated control roots were distinctly different and the analysis could separate them into two distinct groups of treated and untreated (Figure 3.7).

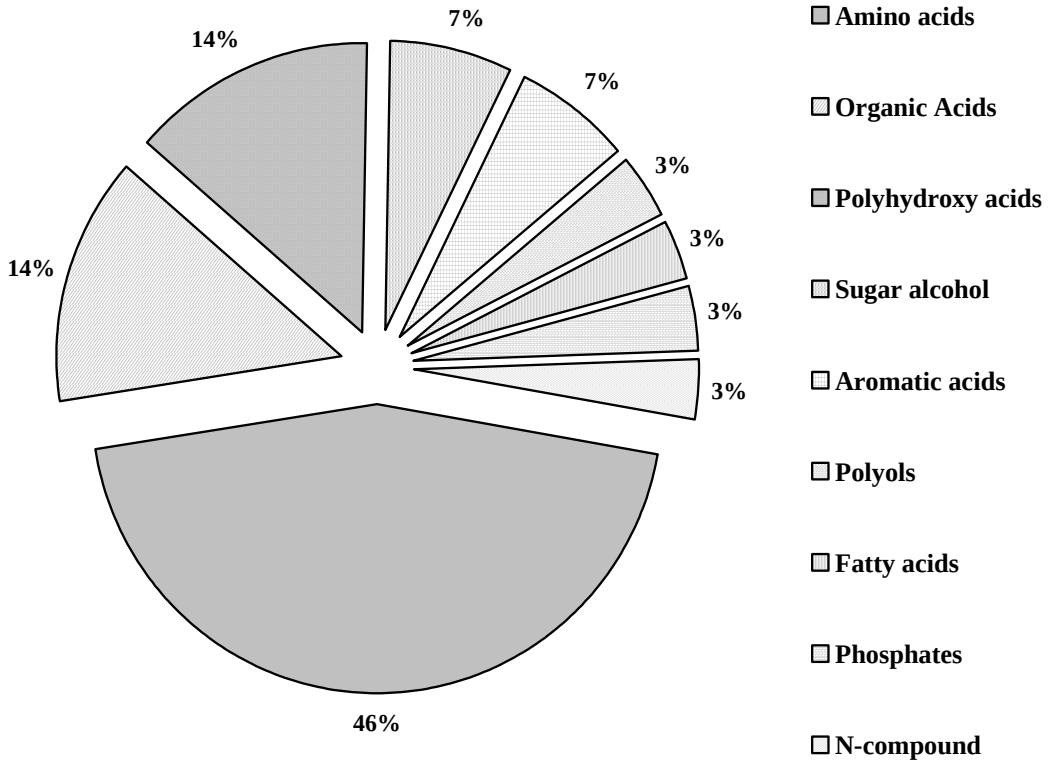


Figure 3.8 Lumichrome induced changes in metabolite classes of *Lotus japonicus* roots. Only levels of metabolites that were significantly different from the untreated control roots were considered.

In the roots, where the growth was enhanced, 46 % of metabolite changes occurred in the amino acid pool upon lumichrome treatment (Figure 3.8). All the amino acids decreased when compared to untreated control roots. Organic acids and polyhydroxy acids each comprised 14 % of the metabolic changes, displaying lower levels in lumichrome treated roots. Generally,

sugar alcohols decreased and constituted 7% of the changes. Aromatic acids too constitute 7% of the changes, displaying increased and decreased levels in lumichrome treated plants. Lastly, decreases in polyols, fatty acids, phosphates and N-compounds each constituted 3% of the metabolite changes.

Considering the specific metabolites, it was apparent that significantly lower levels of metabolites were present in lumichrome treated roots, compared to the untreated control roots (Supplementary data, Table B). Regarding changes in sugar metabolism, glucose decreased and raffinose displayed particularly low levels. The sugar alcohols, erythritol and xylitol, were also decreased. Additionally, the N-compound putrescine was decreased, along with tetradecanoic acid which is involved in lipid metabolism. Abundance levels of organic acids decreased, specifically fumaric acid and malonic acid displayed particularly low levels. Metabolite levels of lumichrome treated roots displayed only two metabolites that were more abundant in lumichrome treated roots, whilst the majority of the metabolites that were significantly different exhibited decreased levels. Elevated levels of benzoic acid and myo-inositol were found in treated roots. Increased levels of myo-inositol are usually associated with stress conditions e.g. increased salinity and heat (Nelson *et al.*, 1998; Brosche *et al.*, 2005). As part of their antimicrobial defence, plants form benzoic acids and other phenylpropanoides in their initial response to infection (Niemann *et al.*, 1991), which might be the reason for increased levels of benzoic acid in this study as the compound lumichrome stems from rhizobacteria.

Of great interest is the finding that the majority of the metabolites that displayed lower levels in lumichrome treated roots were amino acids and organic acids. Specifically, exceptionally low levels of asparagine and arginine were detected. In addition, decreases were observed in the levels of amino acids deriving from oxaloacetate (asparagine, aspartate, lysine, threonine, isoleucine, valine), α-ketoglutarate (arginine, proline) and phosphoglycerate (glycine, serine). The explanation for the decreased amino - and organic acid content can be two-fold. The plant either might be responding to nitrogen deficiency or it might be under oxidative stress.

Firstly, nitrogen deficiency leads to major changes in secondary metabolism. Low nitrogen typically leads to the accumulation of secondary metabolites, including phenylpropanoides and flavonoids (Van der Werf *et al.*, 1993; Gebauer *et al.*, 1998; Hakulinen, 1998; Lou and Baldwin, 2004; Fritz *et al.*, 2006). Central metabolism is an important source of precursors for the synthesis of secondary metabolites which have a range of functions in metabolism,

signaling and defence against abiotic and biotic stress. Alkaloids are synthesized from nitrogen-rich amino acids, phenylpropanoids from phenylalanine by a reaction sequence that leads to the recycling of ammonium, and terpenoids from acetyl CoA or glycolytic intermediates. Some secondary metabolites are carbon-rich e.g. phenylpropanoides, others are nitrogen-rich e.g. alkaloids (Fritz *et al.*, 2006). There is a carbon-nutrient balance hypothesis that postulates that secondary metabolism is directed towards carbon-rich metabolites in nitrogen-limited plants, and nitrogen-rich metabolites in carbon-limited plants (Coley *et al.*, 1985; Van Dam *et al.*, 1996). Whether this is triggered by changes in the level of nitrate, nitrogen metabolism, accompanying changes in carbohydrates or pleiotropic changes resulting from dislocation of cellular functions (Fritz *et al.*, 2006) is not specifically known. In previous studies, nitrogen-depleted plants displayed decreases in amino acids and sugars (Geiger *et al.*, 1998+1999; Matt *et al.*, 2001, Scheible *et al.*, 1997a; Fritz *et al.*, 2006), which is similar to the results in this study. Moreover, secondary metabolism seems to be up-regulated in lumichrome treated plants. Additionally, studies have proven that nitrate modulates shoot-root allocation and lateral root growth (Scheible *et al.*, 1997b; Walch-Liu *et al.*, 2005). Notably, lumichrome significantly increased root growth in treated plants.

The second explanation for decreased amino acid and organic acid content might be that lumichrome treatment might cause the plant to respond as if it were under oxidative stress. Lehmann *et al.* (2009) investigated the response of *Arabidopsis* roots to oxidative stress in a comprehensive and fascinating study. The roots were treated with menadione, which induced the oxidative stress, and the transcriptome, metabolome and key metabolic fluxes were analysed over time. The results showed a decrease in the metabolites of the TCA cycle and amino acid biosynthesis and the transcriptomic response was dominated by the up-regulation of genes for DNA regulatory proteins. These results parallel the results of this study. Changes occurred in stress-associated metabolites that are able to prevent oxidative damage and improve stress tolerance in plants. Regarding polyamines, putrescine showed decreased levels compared to the control plants. Gene expression levels for ornithine decarboxylase, which synthesizes putrescine, were induced in treated plants and therefore transcript and metabolite levels do not coincide. However, spermidine and spermine were not measured and decreased levels of putrescine imply accumulation of both of these metabolites. Of particular note however is that gene for amine oxidase, which degrades polyamines, was up-regulated in treated plants producing H_2O_2. In accordance with the above results, transcripts encoding glutathione S-transferase and peroxidases were up-regulated, providing evidence for the transcriptomic response in the antioxidant defence system. Specifically glutathione S-

transferase is known to be involved in the response towards oxidative stress and detoxification (Dixon *et al.*, 2002). It is important to note that this enzyme was up-regulated in *Arabidopsis* roots under oxidative stress (Lehmann *et al.*, 2009).

In the rhizosphere environment, readily available nutrients are released from the plant in the form of root exudates (Lynch and Whipps, 1990). The presence of microorganisms in the rhizosphere increases root exudation, which contains amino acids, organic acids, proteins, vitamins and other beneficial substances affecting growth and the development of microbial cell population. Lumichrome is a bacterial metabolite from *Sinorhizobium meliloti* and the continuous supply during the treatment might cause an increase in the exudation. This might possibly be an additional explanation for the low levels of organic acids and amino acids found in treated plants. Previous studies have shown that the specific organic acids, amino acids and sugars that displayed decreased levels in lumichrome treated plants, with the exception of cinnamic acid and fucose, have been identified in root exudates (Sundin, 1990; Dakora and Phillips, 2002; Somers and Vanderleyden, 2004). Thus, it might be that the plant perceives lumichrome as a continuous signal for a growing microbial population, consequently increasing the root exudates. However, if this indeed plays a role in the increase in root growth, has to be determined.

Furthermore, metabolite levels of glycerol-3-phosphate were reduced in lumichrome treated roots, compared to the untreated control. Reduced levels of glycerol-3-phosphate were observed in P-deficient plants along with reduced levels of the organic acids fumaric acid, succinate and oxoglutarate (Huang *et al.*, 2008), which was the case in this study. This might indicate that the plant is reacting as if it is under phosphate stress. However, glycerol-3-phosphate is an important metabolite for glycerolipid synthesis, which is a component of all cellular membranes or for the storage of triacylglycerols. During the synthesis of phospholipids, fatty acids are transferred to glycerol-3-phosphate to ultimately produce diacylglycerol where phosphorylated head groups are added to synthesize different classes of phospholipids (Stymme and Stobart, 1987). If the glycerol-3-phoshate is indeed used for this purpose, the higher demand for phosphate to produce cellular membranes as a consequence of growth might alter the phosphate homeostasis in the plant therefore displaying symptoms of P-deficiency.

TARGETED ANALYSIS OF GS1 AND GAPDH

In order to confirm the proteomic results and verify the potential roles of GS1 and GAPDH in the lumichrome mediated growth promotion, a targeted approach utilizing northern blot analysis, western blot analysis and the measurement of enzyme activities was taken.

Cytosolic glutamine synthetase

Northern blot analysis was completed to investigate if the gene expression levels also reflected the proteomic results. Contradictory to the proteomic results, northern blot analysis showed that, compared to the untreated control, glutamine synthetase transcripts were up-regulated in lumichrome treated roots (Figure 3.9 A). This corroborated the microarray results, where a small increase in transcript levels of GS1 was observed. Additionally, the enzyme activities were measured to assess whether the lower abundance of protein too was reflected in the enzyme activities (Figure 3.9 B). There was no significant difference in the enzyme activities of lumichrome treated and untreated roots. Western blot analysis showed that, compared to the untreated control roots, lumichrome treated roots did not show any difference in the protein levels of GS1 (Figure 3.9 C). The possibility might exist that the protein that was identified in the 2-DE gel is one of the isoenzymes with a different migration behaviour and physiological role. The enzyme activities measures GS1 as a whole, not the individual isoenzymes which might differ in activity. Therefore, more detailed analysis is needed to determine the exact role of GS1 in the lumichrome mediated growth promotion.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; phosphorylating NAD-specific)

As with GS1, the northern blot analysis showed a slight increase in transcript levels of GAPDH in lumichrome treated roots (Figure 3.10 A). However, microarray data revealed that there was no significant difference between treated and untreated roots in the gene expression levels of GAPDH. Furthermore, the enzyme activities showed no significant differences between lumichrome treated and untreated roots (Figure 3.10 B). As the protein levels do not parallel those of the RNA and enzyme activity levels, it seems that GAPDH might be under post-translational modification leading to a different migration behavior in the two-dimensional gel. GAPDH has been previously shown to be subjected to translational and multiple post translational control, especially when subjected to stress (Chang *et al.*, 2000; Laukens *et al.*, 2001; Bustos and Iglesias, 2003).

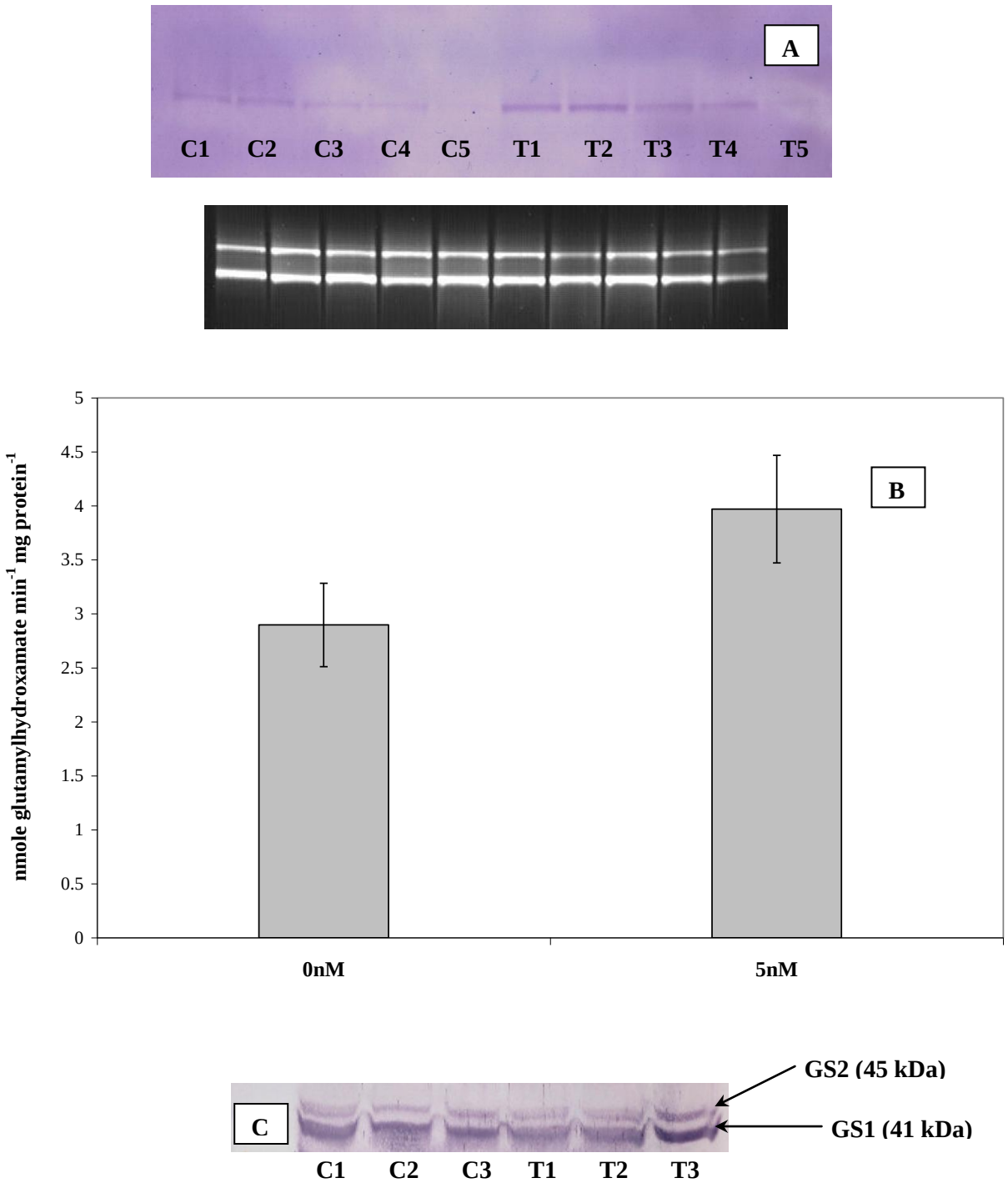
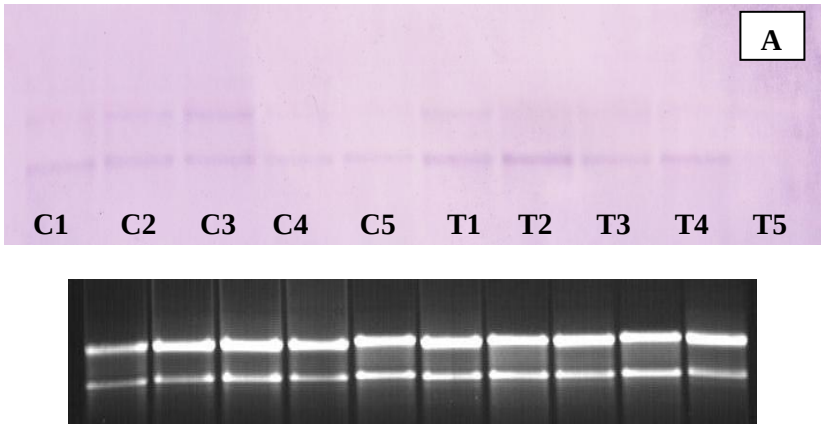


Figure 3.9 Analysis of the effects of lumichrome on glutamine synthetase (GS1) expression in untreated control and lumichrome treated *Lotus japonicus* roots. **A)** Northern blot analysis (top) of transcript levels in treated plants. The gel (bottom) represents the ethidium bromide stained ribosomal subunits that were used to verify equal loading. Lane C1 – C5: untreated control roots; Lane T1 –T5: lumichrome treated roots. **B)** Enzyme activities of treated (5 nM) and untreated (0 nM) roots. Values calculated as mean ± SE of three determinations; n = 5 and significance tested by Student *t*-test ($P < 0.05$). **C)** Western blot analysis of protein abundance of treated and untreated roots; 25 micrograms of soluble protein was loaded into each lane. Lane C1 – C3: untreated control roots; Lane T1 – T3: lumichrome treated roots.



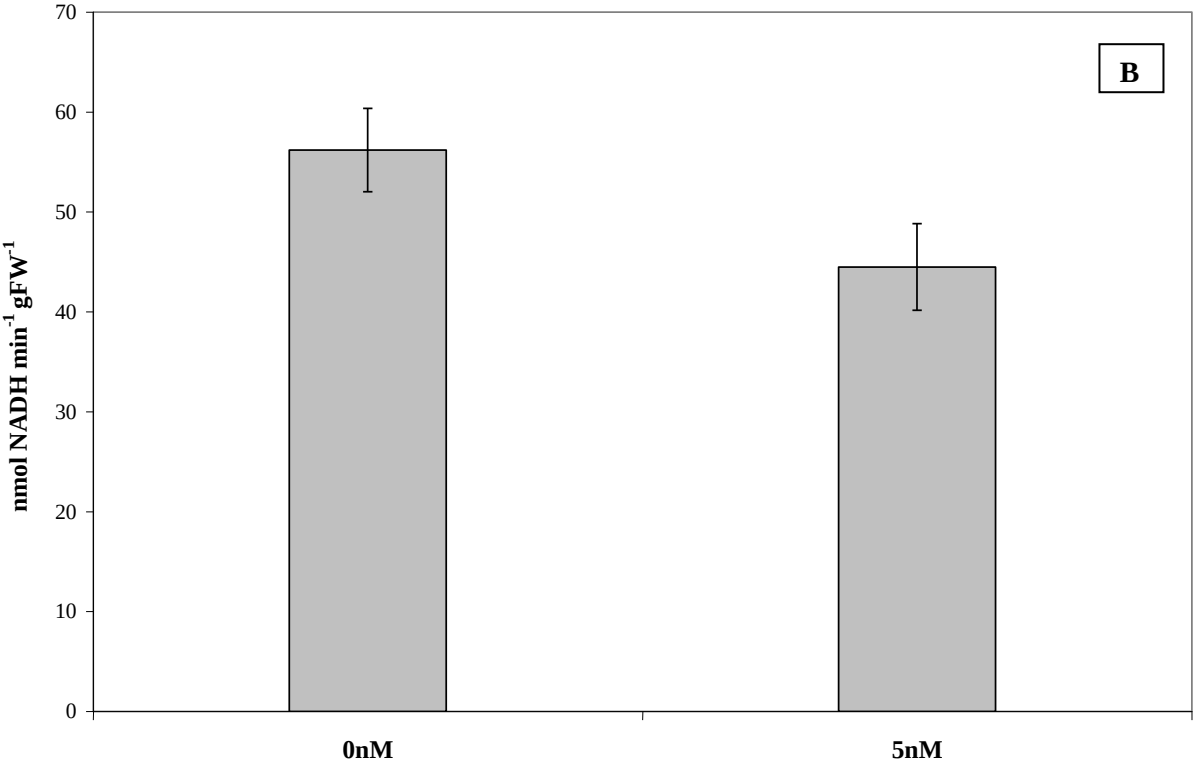


Figure 3.10 Analysis of the effects of lumichrome on glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression in untreated control and lumichrome treated *Lotus japonicus* roots. **A)** Northern blot analysis (top) of transcripts in treated plants. The gel (bottom) represents the ethidium bromide stained ribosomal subunits that were used to verify equal loading. Lane C1 – C5: untreated control roots; Lane T1 –T5: lumichrome treated roots. **B)** Enzyme activities of treated (5 nM) and untreated (0 nM) roots. Values calculated as mean ± SE of three determinations; n = 5 and significance tested by Student *t*-test ($P < 0.05$).

Do H_2O_2 and ABA mediate signaling processes involved in the increased growth response of roots to lumichrome?

The other identified proteins in the comparative 2-DE analysis (Table 1) were also down-regulated. One of these proteins was Fe-superoxide dismutase. The fact that it is down-regulated in lumichrome treated roots, is peculiar as the various responses on lumichrome treatment indicated that the plant is perceiving lumichrome as signal for stress. Usually, reactive oxygen species (ROS)-scavenging enzymes such as superoxide dismutase (SOD) are up-regulated in response to stress conditions as this is the most common mechanism for detoxifying ROS during these stressful periods. However, differential responses in stressed plants have frequently been observed (Kohler *et al.*, 2008). A recent study was performed, where the effects of plant growth promoting rhizobacteria (*Pseudomonas mendocina*) on antioxidant enzyme activities in drought stressed lettuce plants were tested where a pronounced decrease in superoxide dismutase activity was observed (Kohler *et al.*, 2008). In this present study, the plant's response to lumichrome seems to show the involvement of H_2O_2 in reactions such as the up-regulation of the transcription factor MAPK3 and peroxidases. As SOD converts superoxide radicals to H_2O_2 and molecular oxygen, and it is down-regulated in this study, the interesting question arises: if H_2O_2 does in fact play a role in the lumichrome mediated growth promotion, where does the H_2O_2 stem from? An explanation might be the increase in photorespiration, as photorespiratory H_2O_2 production is favoured in situations of low CO_2 availability (Noctor *et al.*, 2000) such as water stress. The treatment with lumichrome has been shown to affect plant water relations through changes in stomatal conductance, therefore this might be possible. Transcripts for MAPK3 and BLH1, which are transcription factors that respond to ABA, were both up-regulated in lumichrome treated plants. Consequently, the involvement of ABA can cause the stomata to close, causing changes in CO_2 availability possibly leading to photorespiration. Transcripts for amine oxidase, which degrades polyamines, were increased upon lumichrome treatment. Interestingly, the degradation leads to an increase in H_2O_2.

CONCLUSION

Lumichrome elicited growth at nanomolar concentrations in *Lotus japonicus* roots. Transcript profiling of *Lotus* roots showed that the three categories that displayed the highest number of differentially expressed genes were RNA regulation of transcription, signaling and stress and defence related genes. The proteomic analysis resulted in the identification of five proteins that were present in lower abundance in lumichrome treated roots. A more targeted analysis

of GS1 and GAPDH was performed to confirm their relevance and importance in the lumichrome mediated growth promotion. Contradictory to the proteomic results, northern blot analysis resulted in a slight increase in transcript levels of GS1 and GAPDH. The microarray data confirmed the result, however, only for GS1. For GAPDH, there were no significant differences present. Enzyme activities for both GS1 and GAPDH showed no significant difference between lumichrome treated and untreated roots. Moreover, western blot analysis for GS1 could not detect significant differences in protein levels. Therefore, one can conclude that GAPDH and GS1 seem to be under post-translational modification leading to a different migration behavior in the 2-D gels; however, further investigation is needed to confirm this statement. In treated plants, most of the metabolite changes occurred in the amino acid pool and point to nitrogen deficiency and oxidative stress. Legumes usually are not colonized by rhizobia when they are supplied with sufficient nitrogen. As the *Lotus* plants were grown under optimal nutrition it might be that lumichrome is perceived as rhizobial elicitor and defence reactions are induced to prevent colonization. Lumichrome perhaps mimics a pathogen attack and therefore might trigger changes in hormone balances which often occur during plant-pathogen interactions (Navarro *et al.*, 2008; Grant and Jones, 2009). These changes in hormone balance might cause the increase in growth. Future experiments will focus on the determination of plant hormone levels after lumichrome treatment.

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SUPPLEMENTARY DATA

DNA MICROARRAY ANALYSIS

Table A *Genes up-regulated in Lotus japonicus roots in response to 5 nM lumichrome treatment*

* Average ratio = ratio of average of each group for each gene, based the ratio if > 2 is up-regulated, < 0.5 is downregulated

**Ts = Student T-test; $P \leq 0.05$

GENE ID AND ANNOTATION	*CATEGORY*	*AVERAGE RATIO**	*TS***
TRANSPORT			
Lotus japonicus similar to At5g61210: Snap25a	Transport – Vesicle trafficking	4.59	0.05
Lotus japonicus similar to At5g28750: Tha4 protein – like	Transport – Protein	2.46	0.04
Lotus japonicus similar to At5g46800: Carnitine acylcarnitine translocase-like protein	Transport – Carrier protein (Mitochondria)	2.33	0.03
Lotus japonicus similar to Q9ATM4: Plasma Membrane Integral Protein ZMPIP2-7	Transport – Aquaporins	3.51	0.04
SIGNALING			

Lotus japonicus similar to At3g45640: Mitogen-activated protein kinase 3	Signaling	3.58	0.04
Lotus japonicus similar to At4g34215: Receptor protein kinase - like protein	Signaling – Receptor kinase	2.05	0.03
Lotus japonicus similar to Q9XED4: Receptor-like Protein Kinase Homolog RK20-1	Signaling – Receptor kinase	2.07	0.04
Lotus japonicus similar to At2g32800: Putative protein kinase	Signaling – Protein kinase	2.10	0.05
Lotus japonicus similar to At4g27300: Putative receptor protein kinase	Signaling – Receptor kinase	2.04	0.02
Lotus japonicus similar to At2g39360: Putative protein kinase	Signaling – Protein kinase	2.27	0.02
Lotus japonicus similar to At3g27560: Putative protein kinase (ATN1)	Signaling – Protein kinase	2.76	0.03
Lotus japonicus similar to At4g08850: Receptor protein kinase like protein	Signaling – Receptor kinase	2.43	0.02
Lotus japonicus similar to O48940: Polyphosphoinositide Binding Protein (SSH2P)	Signaling – Stress response; Osmoprotection	2.19	0.05
CELL WALL			
Lotus japonicus similar to At1g55850: Putative cellulose synthase catalytic subunit	Cell wall – cellulose synthesis	3.41	0.03

Lotus japonicus similar to Q43096: Caffeic Acid O-Methyltransferase	Cell wall – lignin synthesis	2.09	0.03
Lotus japonicus similar to At3g09220: Putative laccase	Cell wall – lignification	2.60	0.05
Lotus japonicus similar to At5g53870: Predicted GPI-anchored protein	Cell wall synthesis	2.24	0.04
STRESS AND DEFENCE RESPONSE			
Lotus japonicus similar to At1g61300: Similar to disease resistance protein	Biotic stress – disease resistance	3.41	0.04
Lotus japonicus similar to Q9FSZ9: Putative Extracellular Dermal Glycoprotein	Abiotic and biotic stress – signal transduction	2.47	0.00
Lotus japonicus similar to At3g20600: Non-race specific disease resistance protein (NDR1)	Biotic stress – disease resistance	2.54	0.04
Lotus japonicus similar to At4g12010: Like disease resistance protein (TMV N-like)	Biotic stress – disease resistance	3.04	0.04
Lotus japonicus similar to At2g47140: Alcohol dehydrogenase like protein	Abiotic stress – anaerobic stress	3.21	0.03
Lotus japonicus similar to At5g41040: N-hydroxycinnamoyl benzoyltransferase-like protein	Defence response – phytoalexins	2.03	0.04
Lotus japonicus similar to At2g35980:	Defence response – pathogenesis related protein	2.08	0.05

Similar to harpin-induced protein hin1 from tobacco *Lotus japonicus similar to At1g09415:* NIMIN-3	SAR; negative regulators	2.53	0.04
AMINO ACID METABOLISM			
Lotus japonicus similar to At4g33760: Aspartate--tRNA ligase like protein	Amino acid activation	3.29	0.03
Lotus japonicus similar to At3g58140: Phenylalanine-tRNA synthetase-like protein	Amino acid activation	3.27	0.05
Lotus japonicus similar to At2g01170: Putative amino acid or GABA permease	Amino acid metabolism	3.21	0.03
Lotus japonicus similar to At5g60540: Imidazoleglycerol-phosphate synthase subunit H – like	Amino acid biosynthesis – histidine	2.10	0.05
Lotus japonicus similar to At1g74040: 2-Isopropylmalate synthase like protein	Amino acid biosynthesis – leucine	2.30	0.02
Lotus japonicus similar to At1g22410: Putative phospho-2-dehydro-3-deoxyheptonate aldolase 1 precursor	Amino acid biosynthesis –chorismate	2.46	0.02
SECONDARY METABOLISM			
Lotus japonicus similar to At2g07050: Cycloartenol synthase	Sterol synthesis	2.61	0.04
Lotus japonicus similar to At5g13930: Chalcone synthase	Flavonoide synthesis	2.25	0.04

Lotus japonicus similar to At2g37040: Phenylalanine ammonia lyase (PAL1)	Phenylalanine metabolism / nitrogen metabolism	3.10	0.02
CARBON AND NITROGEN METABOLISM			
Lotus japonicus similar to Q9XEY7: Trehalase 1 GMTRE1	Carbon metabolism	3.39	0.05
Lotus japonicus similar to At5g37600: Glutamate--ammonia ligase (glutamine synthetase)	Nitrogen metabolism	2.28	0.02
LARGE ENZYME FAMILIES			
Lotus japonicus similar to At2g18980: Peroxidase (ATP22a)	Large enzyme families –Peroxidases	2.20	0.04
Lotus japonicus similar to At1g05260: Putative peroxidase	Large enzyme families –Peroxidases	3.86	0.04
Lotus japonicus similar to Q9MBE5: Cytochrome P450	Large enzyme families – cytochrome P450	2.84	0.05
Lotus japonicus similar to GTXA_TOBAC (P25317): Probable Glutathione S-transferase	Large enzyme families – detoxification	2.72	0.04
Lotus japonicus similar to At2g36780: Putative glucosyl transferase	UDP glucosyl and glucoronyl transferases	3.13	0.04
Lotus japonicus similar to At2g30140: Putative glucosyl transferase	UDP glucosyl and glucoronyl transferases	2.49	0.05

PHYTOHORMONE SYNTHESIS AND RESPONSE			
Lotus japonicus similar to Q43858: 1-Aminocyclopropane 1-carboxylate Synthase (ACC synthase)	Ethylene synthesis	2.13	0.04
Lotus japonicus similar to At4g20880: Ethylene-regulated transcript 2 (ERT2)	Hormone signaling - ethylene	2.56	0.04
Lotus japonicus similar to At5g19040: tRNA isopentenyltransferase -like protein	Cytokinin biosynthesis	2.30	0.03
PHOTOSYNTHESIS			
Lotus japonicus similar to At1g23730: Putative carbonic anhydrase	Photosynthesis, respiration, pH homeostasis and ion transport	3.81	0.04
CELL DIVISION AND DIFFERENTIATION			
Lotus japonicus similar to Q9FUP6: Suspensor-Specific Protein	Cell differentiation	2.77	0.03
Lotus japonicus similar to At3g09920: Putative phosphatidylinositol-4-phosphate 5-kinase	Root growth	2.02	0.03
POLYAMINES			
Lotus japonicus similar to At2g43020: Putative amine oxidase	Catabolism of polyamines	2.84	0.02

Lotus japonicus similar to P93351: Ornithine Decarboxylase	Polyamines synthesis	2.42	0.05
PROTEIN INTERACTION, MODIFICATION AND FOLDING			
Lotus japonicus similar to At5g17710: GrpE protein	Protein folding/hsp	2.97	0.03
Lotus japonicus similar to O24292: CP12 Precursor	Protein-protein interaction; calvin cycle	2.75	0.04
Lotus japonicus similar to At1g21660: Auxilin-like protein	HSP/protein folding	2.05	0.02
Lotus japonicus similar to At5g15080: Serine/threonine specific protein kinase –like	Postranslational modification	3.08	0.04
Lotus japonicus similar to Q9FVI7: Putative Serine Threonine Kinase GDBrPK	Postranslational modification	2.16	0.04
LIPID METABOLISM			
Lotus japonicus similar to At3g09930: Putative lipase acylhydrolase	GDSL-motif lipase	2.01	0.04
Lotus japonicus similar to At1g29670: Lipase hydrolase-like protein	GDSL-motif lipase	2.73	0.03
Lotus japonicus similar to At5g48230: Acetoacetyl-CoA thiolase (AAT1)	Fatty acid metabolism/ secondary metabolism	4.16	0.04
RNA REGULATION OF TRANSCRIPTION			

Lotus japonicus similar to At3g54770: RNA binding protein – like (RNA recognition motif (RRM)-containing protein)	Transcriptional regulation	2.51	0.03
Lotus japonicus similar to At1g76460: Putative RNA-binding protein	Transcriptional regulation	2.16	0.03
Lotus japonicus similar to At5g03740: Histone deacetylase -like protein	Transcription repressor; ABA and stress response	3.37	0.03
Lotus japonicus similar to At4g00220: Lateral Organ Boundries Domain 30 (LOBD30)	Transcription factors – root growth, development	2.97	0.03
Lotus japonicus similar to At1g27730: Salt-tolerance zinc finger protein like	Transcription factor family – Zinc finger	2.04	0.05
Lotus japonicus similar to At1g09530: Putative transcription factor bHLH8 (Phytochrome interacting factor 3,PIF3)	Transcription factor family – bHLH	2.13	0.02
Lotus japonicus similar to At5g65790: Transcription factor-like protein (MYB68)	Transcription factor family – MYB	2.48	0.01
Lotus japonicus similar to At2g22540: Putative MADS-box protein (SVP)	Transcription factor family – Mads-box	2.89	0.01
Lotus japonicus similar to At1g62300: Transcription factor WRKY6	Transcription factor family – WRKY	2.51	0.04
Lotus japonicus similar to At2g35940: Putative homeodomain transcription factor (BLH1)	Transcription factor family – homeodomain	3.34	0.02

Table B ***Down-regulated genes in Lotus japonicus roots in response to 5 nM lumichrome treatment***

* Average ratio = ratio of average of each group for each gene, based the ratio if > 2 is up-regulated,

< 0.5 is downregulated **Ts = Student T-test; *P* = 0.05

Gene ID and Annotation	***Category***	***Average Ratio****	***Ts***
CELL WALL			
Lotus japonicus similar to At1g71380: endo-1,4-Beta-glucanase	Cell wall elongation	0.455	0.02
CARBON AND NITROGEN METABOLISM			
Lotus japonicus similar to At5g20830: Sucrose-UDP glucosyltransferase (Sucrose synthase)	Carbon metabolism	0.488	0.04
SIGNALING			
Lotus japonicus similar to At2g16750: Putative protein kinase	Signaling – Protein kinase	0.306	0.01
SECONDARY METABOLISM			

Lotus japonicus similar to At5g39050: Anthocyanin acyltransferase - like protein	Flavonoide biosynthesis	0.376	0.03
PHYTOHORMONE SYNTHESIS AND RESPONSE			
Lotus japonicus similar to Q9ZWP8: Cytokinin-Specific Binding Protein	Cytokinin control of cell division	0.397	0.01
TRANSPORT			
Lotus japonicus similar to Q9FVE7: Plasma Membrane Ca^{2+}-ATPase	Calcium transport	0.442	0.01
Lotus japonicus similar to At3g21690: Integral membrane protein (MATE EFFLUX protein)	Transport – xenobiotics and secondary metabolites	0.339	0.03

METABOLITE PROFILING

Table C *Changes in metabolite abundance of Lotus japonicus roots as a result of lumichrome treatment*

Up-regulated	***METABOLITE NAME***	***METABOLITE CLASS***	***RESPONSE RATIO* (5 nM/0 nM)***	***T-TEST*****
	Myo-Inositol	Polyols	1.74	0.000
	Benzoic acid	Aromatic Acid	1.69	0.000
Down-regulated	***METABOLITE NAME***	***METABOLITE CLASS***	***RESPONSE RATIO* (5 nM/0 nM)***	***T-TEST*****
	Malonic acid	Organic Acid	0.39	0.000
	Succinic acid	Organic Acid	0.82	0.010
	Fumaric acid	Organic Acid	0.48	0.000
	Butyric acid	Organic Acid	0.61	0.001
	Cinnamic acid	Aromatic Acid	0.40	0.000
	Valine	Amino acid	0.72	0.001
	Isoleucine	Amino acid	0.78	0.002
	Glycine	Amino acid	0.70	0.001
	Proline	Amino acid	0.63	0.000
	Serine	Amino acid	0.60	0.000
	Threonine	Amino acid	0.66	0.000
	Alanine, beta	Amino acid	0.80	0.015

	Aspartic acid	Amino acid	0.68	0.001
	Asparagine	Amino acid	0.29	0.030
	Ornithine	Amino acid	0.42	0.028
	Arginine	Amino acid	0.36	0.005
	Lysine	Amino acid	0.71	0.001
	Tyrosine	Amino acid	0.57	0.002
	Glycerol-3-phosphate	Phosphates	0.71	0.007
	Galactonic acid	Polyhydroxy acid	0.72	0.000
	Glucuronic acid	Polyhydroxy acid	0.78	0.031
	Saccharic acid	Polyhydroxy acid	0.69	0.009
	Threonic acid	Polyhydroxy acid	0.76	0.000
	Fucose	Sugars	0.83	0.001
	Glucose	Sugars	0.63	0.001
	Raffinose	Sugars	0.52	0.044
	Erythritol	Sugar Alcohol	0.89	0.026
	Xylitol	Sugar Alcohol	0.93	0.026
	Tetradecanoic acid	Fatty acids	0.85	0.005
	Putrescine	N-compound	0.81	0.008

* Response ratio = intensity of the mass of the specific metabolite normalized to fresh weight and the internal standard ribitol ** Significant changes were evaluated using t-test ($p \leq 0.05$)

CHAPTER 4

Lumichrome promotes growth of tomato (*Solanum lycopersicum*) roots and induces the expression of orthologous defence-related genes across species*

ABSTRACT

The treatment of tomato plants with nanomolar concentrations of lumichrome caused significant increases in root growth. The treatment with lumichrome caused complex changes in gene expression, affecting mostly genes associated with RNA regulation of transcription, protein synthesis/degradation/modification and stress and defence. Interestingly, three proteins involved in glycolysis were down-regulated. However, changes in transcript levels and the effects of lumichrome treatment on protein and metabolite levels showed very low correlation. GAPDH was investigated in more detail and seem to be under post-translational modification. Lumichrome seems to induce similar changes in the gene expression of six orthologous genes in both lumichrome treated *Lotus* and tomato. These genes all relate to defence and pathogen related responses. These genes will be targeted for further analysis.

Keywords

Plant growth regulator, lumichrome, transcriptomics, proteomics, metabolomics, defence and pathogen responses

* TO BE SUBMITTED FOR PUBLICATION

INTRODUCTION

Plant growth analysis is an essential step in the understanding of plant performance and productivity and may reveal different strategies of plants to cope with their changing environment (El-Lithy *et al.*, 2004), thus furthering our understanding of general growth responses found in most plant species when confronted with a changing environment.

Through powerful signal molecules, rhizobacteria affect fundamental processes in plants. In recent years, a number of novel rhizobial molecules have been identified that positively affect plant growth and development. Previous studies have shown that *Sinorhizobium meliloti,* which form symbiotic relationships with leguminous plants, increases CO_2 availability by enhancing root respiration in alfalfa (Volpin and Phillips, 1998; Phillips *et al.,* 1999). After various experiments, Phillips *et al.* (1999) could show that lumichrome was the active compound and suggested that it represents a previously unrecognized mutualistic signal molecule in the *Sinorhizobium*-alfalfa association. Lumichrome is a common breakdown product of riboflavin and produced by both chemical and biological factors. Various studies on lumichrome have proven its growth promoting effect in the interaction with plants, specifically legumes and cereals (Phillips *et al.*, 1999; Matiru and Dakora, 2005a). In ascidian larval development, lumichrome also appears to serve a developmental signaling role (Tsukamoto *et al.*, 1999) suggesting that it might act as a universal growth promoter and developmental signal in animals as well as plants. The mode of action, however, is still unknown and remains to be elucidated.

The growth promoting effect of lumichrome in other dicotyledonous crop species, specifically tomato, has not been tested and it remains unclear whether lumichrome has the same advantageous effect. The fact that various biological processes, such as fruit development (Giovannoni, 2004) and plant defence (Gebhardt and Volkonen, 2001) have been studied in tomato as well as the availability of rich genetic and genomic resources (Lee *et al.*, 2007) makes it one of the well suited model systems. After establishing that lumichrome also promotes growth of tomato plants the most important genes, proteins and metabolites affected upon lumichrome treatment were investigated in order to gain more insight into the molecular mechanism of lumichrome.

MATERIAL AND METHODS

Growth studies and plant material

Solanum lycopersicum seeds (cv. Moneymaker) were sterilized and sown in pots containing a mixture of 1:3 (v/v) sand and vermiculite. Pots were initially covered with transparent plastic wrap for approximately six days. Plants were watered every second day with half-strength Hoagland solution (Sigma Aldrich, St. Louis, Missouri, USA) containing 1 mM NH_4NO_3 and the effects of lumichrome on plant growth tested by adjusting the solution to contain 0 nM (control) and 5 nM lumichrome, as described by Phillips *et al.* (1999). The experiments were carried out in the glasshouse of the Institute for Plant Biotechnology during the course of the doctoral study under uncontrolled conditions of light, temperature and humidity. Plants were harvested after five weeks and ground to a fine powder in liquid nitrogen. Samples were stored at -80°C until used.

Transcript profiling

RNA extraction

Total RNA was isolated according to a modified CTAB method of Chang *et al.* (1993). 200 mg of root material was extracted with 1.2 ml of CTAB buffer (2 % (w/v) CTAB, 2% (w/v) PVP, 100 mM Tris-HCl (pH 8.0), 25 mM EDTA, 2 M NaCl, 0.5 g/L spermidine) vortexed immediately and incubated at 65°C for 20 minutes. Samples were centrifuged at 13 000 rpm for 10 minutes and the supernatant transferred to a new tube. A chloroform:isoamylalcohol (24:1) extraction was conducted twice. RNA was precipitated overnight at 4°C by adding 8M $LiCl_2$ to a final concentration of 2M and pelleted the following day by centrifugation at 13 000 rpm for 60 minutes at 4°C. The pellet was resuspended in 50 µl of water. Subsequently, the RNA was quantified spectrophotometrically and the quality visualized by loading five µg on an ethidium bromide stained 1.2% (w/v) agarose gel. Following this, the RNA was treated with DNase I (Promega, Madison, WI, USA) according to the manufacturers' instructions followed by precipitation with 5µl 3M sodium acetate (pH 5.5) and 200 µl 100% ethanol at -20°C overnight. The RNA was pelleted at 13 000 rpm for 40 minutes at 4°C, washed with 70% ethanol and redissolved in 45 µl milliQ water. Consequently, the RNA was column cleaned with the RNA MinElute Cleanup Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions and quantified on the Nanodrop (Thermo Fisher Scientific, USA).

Gene chip microarray experiments and data Analysis

Fifteen micrograms of total RNA was used as template for amplification. Three biological replicates per treatment were hybridized. Labelling and hybridisation of the TOM1 array was followed directly as previously described (Alba *et al.*, 2004), using NCSU as the blocking agent. The TOM1 arrays (Cornell University, http://ted.bti.cornell.edu/) were hybridized and scanned with a Fuji MAS FLA-8000 microarray scanner. The GeneSpotter software (MicroDiscovery) was used for the grid positioning and signal quantification. The resulting data were analyzed using the LIMMA package (Smyth, 2005) for the Bioconductor software (Gentleman *et al.*, 2004). Data were normalized using within-array print-tip Loess and between array quantile normalization. P values were corrected using a FDR correction (Benjamini and Hochberg, 1995). A FDR P value (q value) of < 0.05 was deemed significant. Data were visualized with the MapMan software (Usadel *et al.*, 2005).

MAPMAN analyses

The 34 MapMan BINs used for the *Arabidopsis* MapMan classification (Usadel *et al.*, 2005, Thimm *et al.*, 2004), have been adopted for tomato as defined in Urbanczyk-Wochniak *et al.* (2006). For visualization, the data was loaded into MapMan which displays individual genes mapped on their pathways as false colour coded rectangles. The software can be downloaded, as well as help obtained, from http://gabi.rzpd.de/projects/MapMan. To facilitate comparison of the different colours a legend explaining the changes is included by MapMan, which associates the colour representation with the $\log_2$ fold changes in expression. Since MapMan uses an ontology to display data, it sorts data by biological processes and displays them in a group wise format.

Proteomic analysis

Protein extraction

Approximately 150 mg of root material of each plant was weighed and pooled in pairs resulting in a final amount of 300 mg root material and six samples (three treated, three untreated). Phenol extraction of proteins was performed according to the modified protocol of Hurkman and Tanaka (1986). Approximately 900 µl of extraction buffer (0.1M Tris-HCl pH 8.8, 10 mM EDTA, 0.4% β-mercaptoethanol, 0.9 M sucrose) was added to 300 mg root material, vortexed thoroughly for 30 seconds. Following this, 900 µl of phenol (Tris buffered; pH 8.0) was added and samples were vortexed for an additional 30 seconds and agitated for

30 minutes at 4°C. The phenol phase was separated at 5 000 g for 10 minutes at 4°C. The top phenol phase was removed and placed into a new tube. An equal volume of fresh Tris buffered phenol was added to the aqueous phase, vortexed for 30 seconds and agitated for 30 minutes at 4°C. Again, the phenol phase was separated at 5000 x g for 10 minutes at 4°C and the top phenol phase removed and added to the previous fraction. This fraction was back extracted with an equal volume of extraction buffer where it was agitated for 15 minutes at 4°C, the phenol phase then separated at 5000 x g for 10 minutes and the final phenol fraction transferred to a new tube. Phenol extracted proteins were precipitated by adding 6 volumes of 0.1 M ammonium acetate in 100% (v/v) methanol (prechilled at – 80°C), vortexed thoroughly and incubated overnight at – 80°C. The precipitate was collected by centrifugation at 4 000 x g for 30 minutes at 4°C and the pellet washed twice with ice-cold 0.1 M ammonium acetate in methanol containing 10 mM DTT. The pellet was resuspended in a buffer containing 0.7 M urea, 2.0 M thiourea, 4 % CHAPS and 10 mM DTT.

Two dimensional gel electrophoresis

In preparation for the first dimension, the samples were cleaned with the 2D Cleanup Kit according to the manufacturer's instructions. The protein pellet was resuspended in 600 µl of rehydration/sample buffer, which was the appropriate amount to load 150 µg of protein per 11 cm IPG strip (pH 3 – 10) resulting in three technical replicates per biological sample. The IPG strips were rehydrated overnight and focused using the Protean IEF Cell at 20°C, applying the following program: a linear increase from 0 – 250 V over 20 minutes, 250 V to 8 000 V over 2.5 h and then held at 8 000 V for 20 000 V-hr. In preparation for the second dimension, each strip was equilibrated in 4 ml of Equilibration Buffer I for 10 minutes, thereafter in Equilibration Buffer II for 10 minutes. Each strip was transferred to a 10 % Precast Criterion Tris-HCl gel for the second dimension electrophoresis in the Criterion Cell, MOPS buffer added and 200 V applied for approximately 50 minutes. All three technical replicates were run simultaneously to ensure that the same conditions were maintained. Gels were silver stained according the manufacturer's instructions and scanned with a Hewlett Packard scanner in 600 dpi. Ten prominent, differentially expressed protein spots that could be visually distinguished from those that showed little or no differential expression, were picked. All equipment, chemicals and kits were purchased from Bio-Rad (Bio-Rad, Hercules, CA, USA).

In-gel digestion

Protein spots were in-gel enzymatically digested in a similar manner as previously described by Shevchenko *et al.* (1996) using modified sequence grade porcine trypsin obtained from

Promega (Madison, WI, USA). Silver stained (mass spectrometry compatible) gel pieces were cut from the gel and transferred to an Eppendorf tube. Excess water was removed and 35 µl acetonitrile added to cover gel pieces, then incubated for 10 minutes at room temperature to dehydrate and shrink gel pieces. Acetonitrile was removed and the samples dried under vacuum for 10 minutes. Gel particles were swelled in 150 µl 10 mM DTT in 100 mM NH_4HCO_3, incubated for 1 h at 56°C and then cooled to RT. The DTT solution was replaced with 150 µl 55 mM iodoacetamide in 100 mM NH_4HCO_3, incubated in the dark with occasional vortexing for 45 minutes at RT. The solution was removed; gel pieces were washed with 150 µl 100 mM NH_4HCO_3 and incubate again for 10 minutes at RT. The NH_4HCO_3 solution was removed, 150 µl acetonitrile added to dehydrate gel pieces and incubated for 10 minutes at RT. After repeating the last two steps, the acetonitrile was removed and speed-vac to dryness for 10 minutes. The samples were placed on ice, the particles were swelled in 35 µl digestion buffer (12.5 ng/µl trypsin in 50 mM NH_4HCO_3) and incubated on ice for 45 minutes. Thereafter the trypsin-containing buffer was removed, 10 µl 50 mM NH_4HCO_3 (without trypsin) added to keep pieces wet during cleavage and incubated overnight at 37°C.

The following day the samples were centrifuged for 1 minute at 13 000 rpm and the supernatant kept in a separate PCR tube (Tube 1). To the remaining gel pieces, 20 µl of 20 mM NH_4HCO_3 was added, incubated for 10 minutes at RT and the supernatant transferred to the PCR tube (Tube 1) from the previous step. To the remaining gel pieces, 25 µl of 5 % (v/v) formic acid, 50% (v/v) acetonitrile was added and incubated for 20 minutes at RT, centrifuged for 1 minute at 13 000 rpm and the formic acid/acetonitrile solution was removed and saved in the PCR tube (Tube 1). The acid extraction was repeated twice and lastly the PCR tube (Tube 1) was vacuum dried and stored at -20°C until analysis with CapLC-ESI Q-TOF-MS/MS (Waters) and confirmation with MALDI-MS.

Metabolite profiling

Primary metabolites were extracted and analysed as described by Roessner *et al.* (2001) optimised for tomato following the method of Roessner-Tunali *et al.* (2003). Gas chromatography coupled to electron impact ionization/time-of-flight mass spectrometry (GC/EI TOF-MS) was performed using an Agilent 6890N24 gas chromatograph (Agilent Technologies, Palo Alto, CA) with split or splitless injection connected to a Pegasus III time-of-flight mass spectrometer (LECO Instrumente GmbH) run and evaluated according to Erban

et al., (2007) using TagFinder 4.0 software (Luedemann *et al.*, 2008). Chromatograms and mass spectra were evaluated using Chroma TOF 1.6 and TagFinder 4.0 software (Luedemann *et al.*, 2008). The peak height representing arbitrary mass spectral ion currents was normalized using the sample fresh weight and ribitol content for internal standardization. Metabolites were identified using NIST05 software and the mass spectral and retention time index (RI) collection of the Golm metabolome database (Kopka *et al.*, 2005; Schauer *et al.*, 2005). Significance levels of metabolite changes between lumichrome treated and control roots were evaluated by Student t-test ($P \leq 0.05$). Principal component analysis (PCA) was completed with Statistica 8 © StatSoft.

Northern blot analysis

Total RNA was isolated according to a modified CTAB method of Chang *et al.* (1993) (see Transcript profiling). A probe constructed for *Lotus japonicus* in previous experiments was used, as there is an 80% homology between the sequences. cDNA was reverse transcribed from total RNA using SuperScript III Reverse Transcriptase (Invitrogen corporation, Carlsbad, California, USA) according to the manufacturer's instructions. PCR amplification of the glyceraldehyde-3-phosphate dehydrogenase gene was achieved using 1 unit Taq DNA polymerase, 0.5 mM $MgCl_2$, 0.2 mM dNTP's, 1 x buffer, 0.2 µM Forward primer (ATGGGCAAGATCAAGATCGG), 0.2 µM Reverse primer (CACAGTAGGAACACGGAATG) and 1 µl cDNA reaction template in a final volume of 50 µl. PCR conditions were as follows: 5 minutes denaturation at 94°C; 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute and 30 seconds; final elongation step at 72°C for 7 minutes. The resulting amplicon of 720 bp was separated on a 1% (w/v) agarose gel, the band excised and purified with the PCR purification Kit according to manufacturer's instructions (Qiagen, www.qiagen.com). The fragment was subjected to restriction digest to verify that the right fragment was amplified. For additional verification, the amplicon was cloned into pGEM®-T Easy (Promega, Madison, WI, USA) and sequenced. PCR labeling of the probe was achieved by using the PCR DIG Probe Synthesis Kit according the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). The probe labeling efficiency was checked by running the labeled, unlabeled and positive control on a 1% (w/v) agarose gel, where the labeled probe migrates slower than the unlabeled due to the presence of DIG. For the gel blot membranes, six µg was denatured in formamide with loading buffer and run on a 0.8% (w/v) Tris-Borate/EDTA (TBE) prepared agarose gel. RNA was transferred on a positively charged nylon membrane (Roche Diagnostics, Mannheim, Germany) by upward capillary blotting (Sambrook *et al.* 1989) using 10xSSC (standard saline

citrate). RNA was UV cross-linked for 2.5 minutes at 120 mJ/cm using a UV cross-linker. All hybridisation and washing procedures were carried out as described by the DIG Application Manual for Filter Hybridisation (Roche Diagnostics, Mannheim, Germany).

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) assay

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity was measured according to Velasco *et al.* (1994) modified to microtiter plate format. For the protein extraction, 200 mg root material was resuspended in 800 µl extraction buffer (50 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 1 mM EDTA, 0.1% (v/v) β-mercaptoethanol, 20% (v/v) glycerol). The homogenate was cleared by centrifugation at 13 000 rpm for 15 minutes at 4°C and desalted on Sephadex G-25 (Sigma, St. Louis, Missouri, USA) (medium) using the extraction buffer, omitting β-mercaptoethanol. The assay mixture contained 100 mM Tris-HCl (pH7.6), 10 mM $MgCl_2$, 0.1% (v/v) β-mercaptoethanol, 5 mM ATP, 0.2 mM NADH and 20 units phosphoglycerate kinase. GAPDH was assayed with 100 µl root extract. The reaction was started by adding 4 mM 3-phosphoglycerate. GAPDH activity was determined by measuring the decrease in the concentration of NADH at 340 nm for 5 minutes at room temperature.

RESULTS AND DISCUSSION

PLANT GROWTH STUDIES

Lumichrome significantly increases root biomass in tomato

As previous studies have reported a growth promoting effect at 5 nM lumichrome (Phillips *et al.*, 1999; Matiru and Dakora, 2005a; Khan *et al.*, 2008), this concentration was chosen for the application. The application of 5 nM lumichrome markedly altered the growth of tomato. Where the shoot biomass was not significantly affected (Figure 4.1 A), the roots showed a significant increase in biomass compared to the untreated control (Figure 4.1 B). The increased growth was especially prominent when the lateral roots were examined.

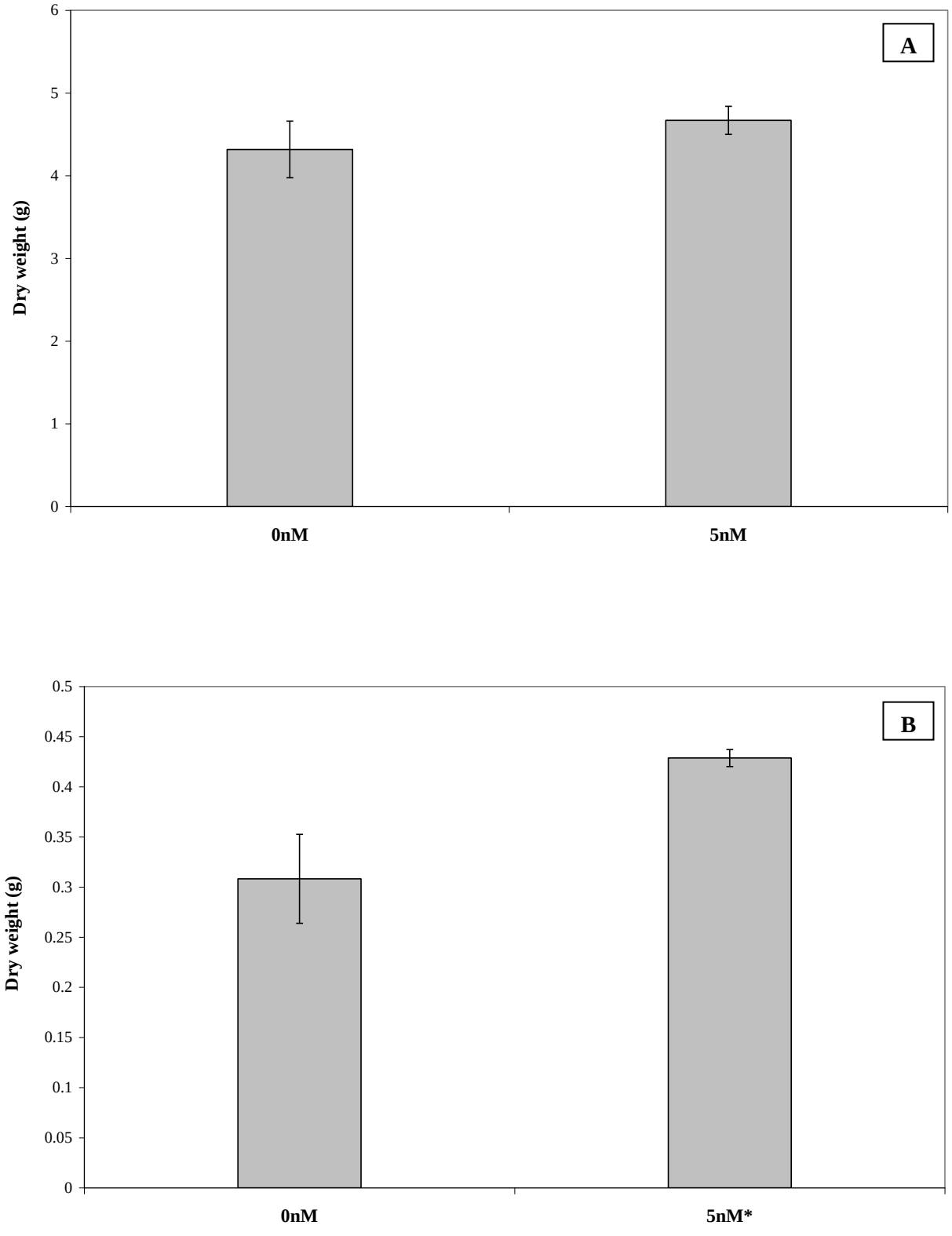


Figure 4.1 The physiological effects of lumichrome on the A) shoot and B) root growth of five week old tomato plants. Mean values (± SE) are reported for five independent replicates. * Indicate treatment effects statistically significant at $P \leq 0.05$, determined by Student t-test.

TRANSCRIPT PROFILING

Transcript profiling of tomato roots in response to lumichrome

In response to lumichrome, the microarray analysis resulted in 769 genes being differentially expressed, after applying a significance filter of $p < 0.05$. From the 769 that were differentially expressed, 452 genes were up-regulated and 317 genes were down-regulated. The microarray results were displayed using the data visualization tool MapMan (Thimm *et al.*, 2004). Among the differentially expressed genes, 493 genes could not be assigned to previously defined MapMan ontologies and this group includes those genes annotated to unknown or hypothetical proteins. The results are presented according to the functional annotation and diagrams visualized through the MapMan software. A complete data set of significantly altered transcripts presented in this section is available as supplementary material (Supplementary data, Table A). An overview of gene expression categorized in functional groups provides information as to which biological process was most affected by lumichrome. Differentially expressed genes were categorically divided (Figure 4.2) and the number of genes induced or repressed upon lumichrome treatment were specified. RNA regulation of transcription, protein synthesis/degradation/modification and stress and defence were the top three categories that showed the most changes regarding differential gene expression.

General metabolism

In glycolysis (Figure 4.3), the gene coding for phosphoenolpyruvate carboxylase (PEPC) was induced, whilst the gene annotated to enolase (phosphopyruvate dehydratase), which catalyses the reversible reaction between 2-phosphoglycerate and phosphoenolpyruvate (PEP) in the lower part of the glycolytic pathway, was suppressed upon treatment with lumichrome. It is important to note that PEPC activity reflects the relationship between nitrogen and carbon metabolism (Champigny and Foyer, 1992). It has been shown that roots under phosphate stress tend to markedly induce PEPC, which has been correlated with the secretion of significant levels of organic acids such as malate and citrate. The reason for this being is that this step leads to the acidification of the rhizosphere and therefore contributes to the solubilization and assimilation of mineral Pi from the environment (Plaxton, 2004). In the glyoxylate cycle (Figure 4.3), where the major purpose of the cycle is to convert acetyl-CoA from fatty acid degradation into malate (for gluconeogenesis), the gene annotated to citrate synthase was induced.

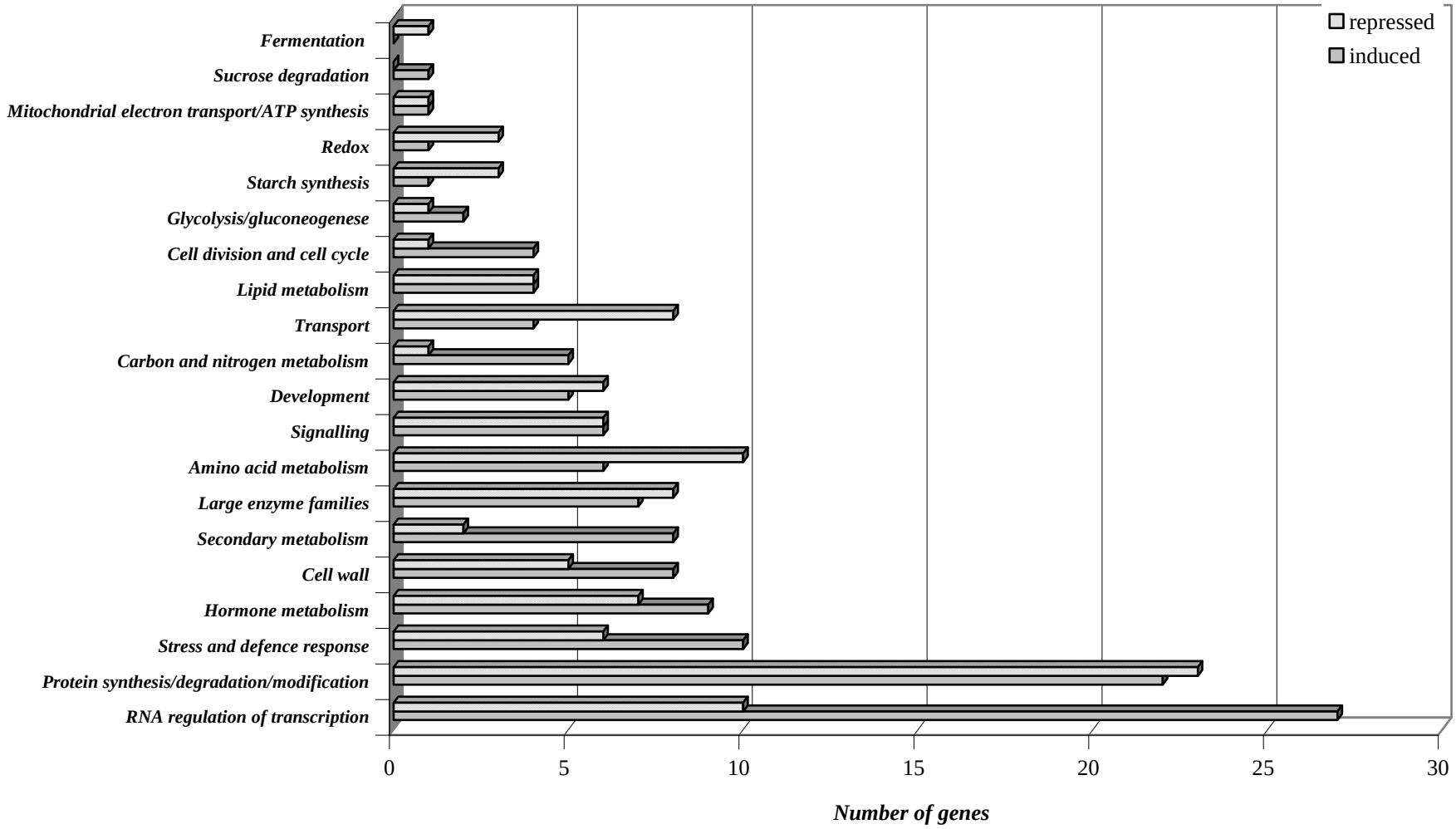


Figure 4.2 Schematic representation of significantly induced or repressed transcripts in lumichrome treated tomato roots grouped according to gene ontology. Genes of unknown function or classification are not shown. The number of genes found in each category is identified on the *x*-axis. The values are representative of the log2 transformed values of three individual hybridisations, statistically analysed and Loewess tip-point normalised in R, and classed according to the MAPMAN classifications (Thimm *et al.*, 2004; Usadel *et al.*, 2005) adapted for tomato (Urbanczyk-Wochniak *et al.*, 2006). For information on individual genes, see Table A of the supplementary data

Citrate synthase catalyses the first reaction in the cycle where acetyl-CoA is converted to citrate. Previous studies have shown that enolase, together with phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase, fructose-1,6-bisphosphate aldolase, pyruvate kinase, triose phosphate isomerase and hexokinase associate with the outer mitochondrial membrane (Giegé *et al.*, 2003; Graham *et al.*, 2007). It is suggested that this protein forms part of the partial glycolytic pathway at the mitochondria directs carbon towards respiration (Graham *et al.*, 2007). Taken together, these changes in genes involved in glycolysis and/or organic acid metabolism are consistent with the proposed role of lumichrome in enhancing respiratory activity in roots (Phillips *et al.*, 1999).

Generally, with a few exceptions, there was a decrease in transcripts for enzymes involved in amino acid synthesis (Figure 4.3) and an increase in transcripts encoding enzymes involved in amino acid breakdown. Genes for the synthesis of proline, methionine and cysteine were strongly suppressed as well as for phenylalanine. Proline has an influence on various processes during stress conditions in plants i.e. energy-related pathways and carbon metabolism. The gene coding for dehydroquinate synthase, which is responsible for chorismate synthesis, was strongly suppressed. One exception is the up-regulation of the gene annotated to tryptophan synthase. Genes annotated to the central amino acid metabolism, specifically the gene coding for glutamate decarboxylase (GAD), was highly up-regulated in lumichrome treated plants. Glutamate decarboxylase catalyzes the conversion of glutamate to γ-aminobutyric acid (GABA), a non-protein amino acid. With regards to degradation of amino acids, there was a strong increase in genes for histidine degradation; however a decrease in the genes for degradation of the glutamate family of amino acids. Wang *et al.* (2000) performed studies on nitrate induced genes in *Arabidopsis thaliana* and among the genes that were induced, was histidine decarboxylase. This might be an indication that the nitrogen metabolism is affected by lumichrome treatment.

Carbon and nitrogen metabolism

Genes assigned to sucrose and starch metabolism (Figure 4.3) unquestionably responded to lumichrome treatment. Generally, genes for sucrose degradation increased whilst genes for starch degradation decreased. Regarding starch synthesis, the gene annotated to starch synthase II was down-regulated, however there were no consistant changes in the gene expression of the various subunits of ADP-glucose pyrophosphorylase. Nitrogen is the most important inorganic nutrient in plants and a major component of proteins, nucleic acids, many cofactors and secondary metabolites (Marschner, 1995).

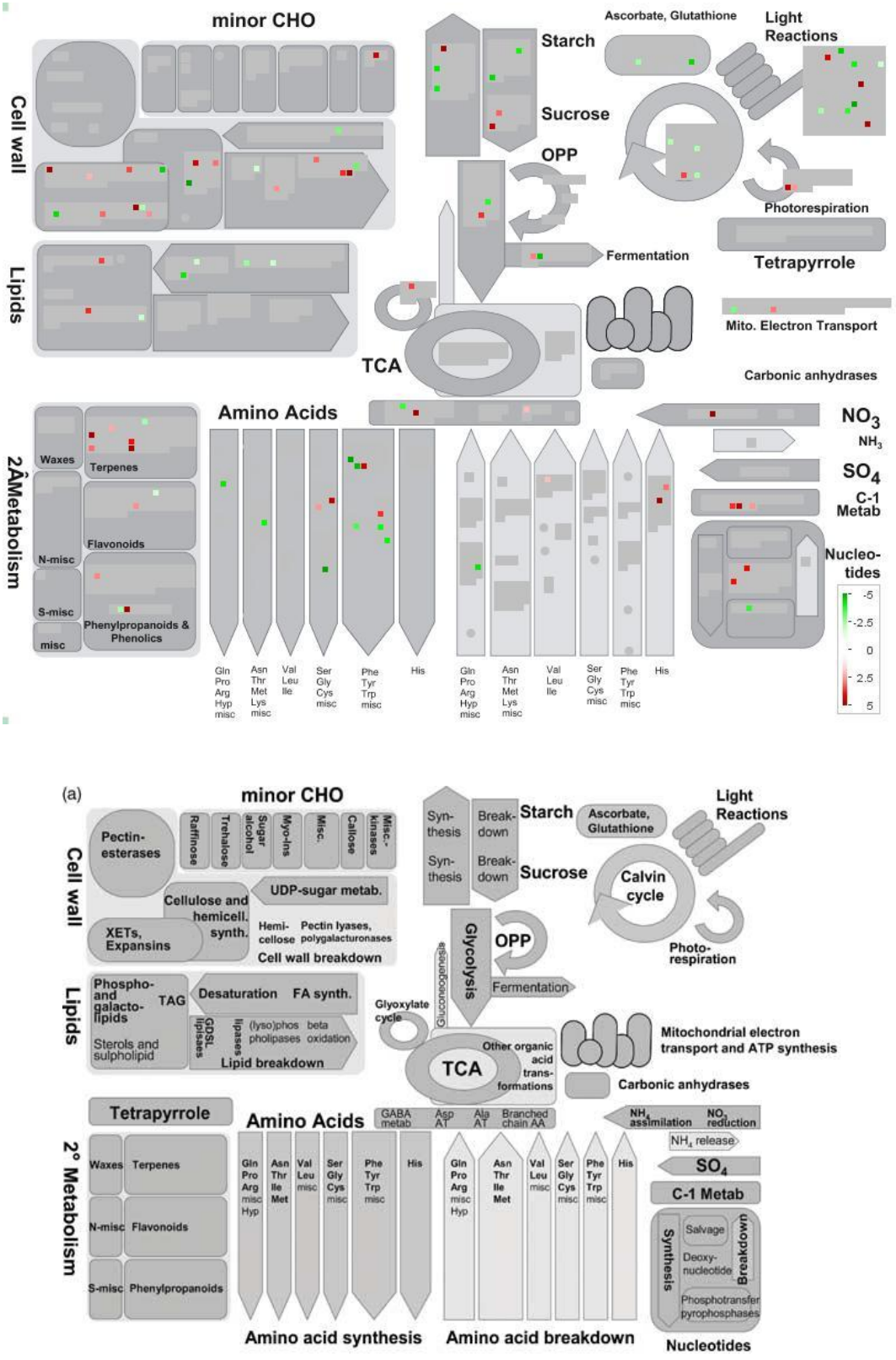


Figure 4.3 MapMan visualization of metabolism-related gene expression. Genes were assigned to their associated metabolic pathway. Red represents genes that are induced, green represents genes that are repressed $P = (< 0.05)$. The magnitude of change is indicated in the colour scale represented in the figure (between +5 and -5). a) Explanation of the functional categories deposited at each location in the figure.

Nitrogen affects all levels of plant function, from metabolism to resource allocation growth and development (Marschner, 1995; Crawford, 1995). Lumichrome treatment seems to have strongly affected nitrogen metabolism (Figure 4.3) in that the genes for glutamate synthase and glutamate decarboxylase, which catalyzes the conversion of glutamate to GABA, was up-regulated. Activated by Ca^{2+}/calmodulin, GABA synthesis in plants is involved in various processes such as pH regulation, the storage of nitrogen, plant development and defence, plays a role as a compatible osmolyte and lastly is an alternative pathway for glutamate utilization (Shelp *et al.*, 1999). A study by Ford *et al.* (1996) showed that during the process of hormone induced (NAA and kinetin) dedifferentiation of root tissue, GABA levels were enhanced while glutamate levels were reduced. GABA, specifically, can act as a shoot-to-root signal regulating nitrate uptake and the balance between carbon and nitrogen in plants. This role has also been suggested for glutamine (Beuve *et al.*, 2004), which displayed increased levels in lumichrome treated plants in this study (see below).

Cell wall

A large group of genes, belonging to the functional group cell wall metabolism (Figure 4.3), showed differential expression. Nearly all of the genes annotated as coding for cell wall pectin esterases increased. Expression of genes encoding proteins involved in cell wall modification showed increases in xyloglucan endotransglucosylase-hydrolase 5 (XTH5) and a decrease in XTH7. Xyloglucans are seen as the most important polysaccharide controlling cell wall expansion and the XTH proteins are potentially involved in wall loosening to enable turgor-driven expansion or they contribute to wall biogenesis and catalyse the incorporation of new cell wall material. BRU1, which is a brassinosteroid-regulated XTH protein, was strongly induced in lumichrome treated plants. It has been shown that the transcript levels of several XTHs from different plant species are up-regulated in response to brassinosteroids (Müssig and Altmann, 2003). Nearly all the genes annotated to encoding proteins for cell wall degradation enzymes were induced among treatment with lumichrome. Moreover, the gene annotated to encode callose synthase was induced whilst the gene encoding cellulose synthase was strongly repressed. Interestingly, Ton and Mauch-Mani (2004) found in their study that the induction of resistance mediated by the non-protein amino acid β-aminobutyric acid is based on the ABA-dependant priming of callose. Possibly the increased expression of callose synthase might point to increased induction of disease resistance and the potential role of ABA in signaling in this process.

Secondary and lipid metabolism

Regarding secondary metabolism (Figure 4.3), there seems to be a strong up-regulation of genes that encode enzymes for carotenoid synthesis, specifically those annotated to encode phytoene synthase, phytoene desaturase and mevalonate kinase. There was a strong induction of genes for the mevalonate pathway. This is important to note, as the mevalonate pathway is the main route to the production of steroidal backbones (Benveniste, 2004; Schaller, 2004) and might point to the synthesis of brassinosteroids. There was a strong increase in the gene expression of the gene annotated to laccase, which is involved in the lignification of cell walls (Mayera and Staples, 2002). Various genes encoding enzymes belonging to the family of cytochrome P450 monooxygenases were induced, particularly the gene annotated to encode flavonoid 3',5'-hydroxylase, indicating possible increases in flavonoid biosynthesis. The impact of lumichrome on lipid metabolism (Figure 4.3) in tomato roots was reflected in increases in the expression of genes encoding proteins involved in phospholipid synthesis but decreases in genes encoding enzymes involved in fatty acid desaturation as well as fatty acid synthesis and elongation. Additionally, genes annotated to encode lipases were up-regulated in lumichrome treated plants, which play a role in lipid degradation where they catalyze the hydrolysis of fatty acids from the glycerol backbone.

Cell division and cell cycle

MapMan assisted analysis of tomato roots treated with lumichrome displayed a strong induction of genes encoding proteins involved in cell division and especially the cell cycle (Figure 4.4), which included a strong induction of the gene for Peptidyl-prolyl cis-trans isomerase (PPIase) and the up-regulation of the gene for CDK-activating kinase. PPIases interconverts the cis and trans isomers of peptide bonds with the amino acid proline. The process of cis-trans isomerization is often the rate-limiting step in the process of protein folding. Prolyl isomerases therefore function as protein folding chaperones. The *cis-trans* interconversion accelerated by PPIases is of importance for the final protein structure because *cis* proline introduces bends within the protein (Harrar *et al.*, 2001). *Arabidopsis thaliana* plants that were subjected to H_2O_2 stress exhibited high transcript levels of a gene annotated to PPIase (Davletova *et al.*, 2005), which might indicate the possibility that the lumichrome treated plants are responding as if they were under H_2O_2 stress.

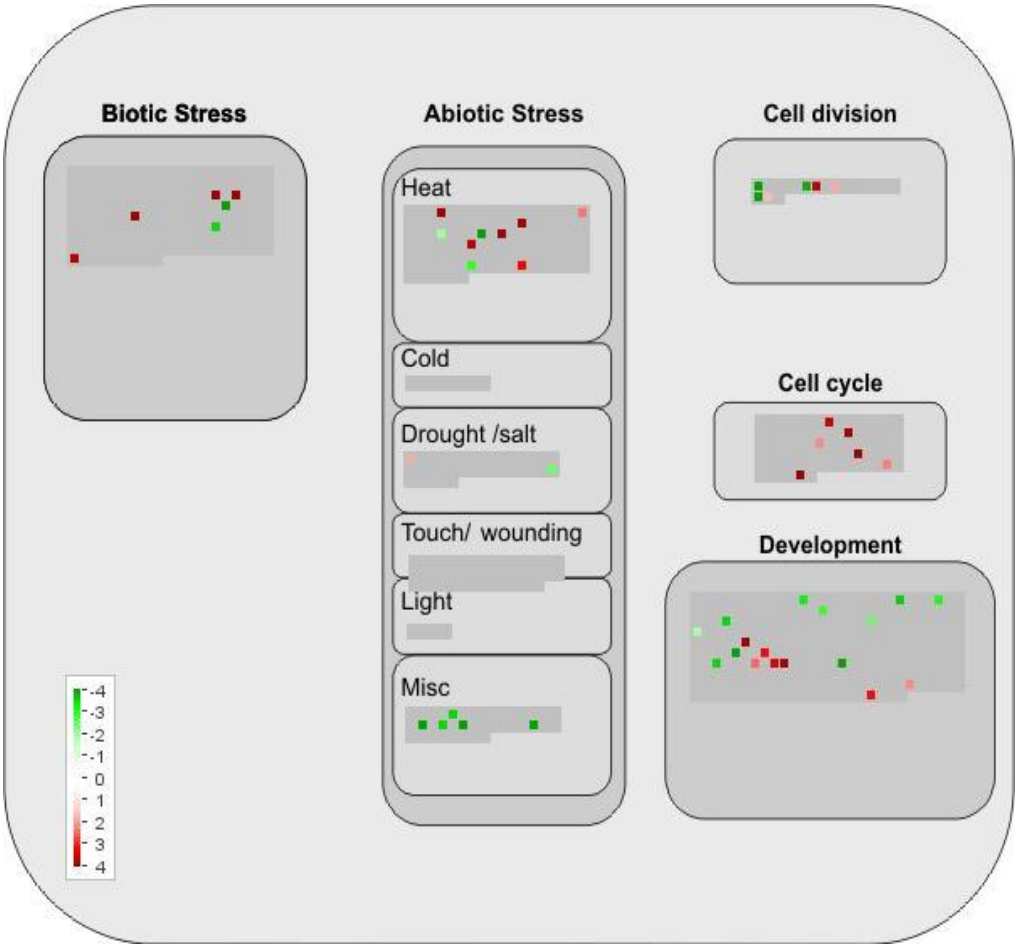


Figure 4.4 MapMan visualization of genes annotated to cellular response that showed differential expression *P* = (< 0.05). Red represents genes that are induced, green represents genes that are repressed. The magnitude of change is indicated in the color scale represented in the figure (between +5 and -5).

Stress and defence

Regarding the biotic stress response (Figure 4.4), mostly genes encoding disease resistance proteins were induced. The abiotic stress response (Figure 4.4) revealed a strong up-regulation of genes coding for members of the heat shock transcription factor family but the down-regulation of genes coding for the calmodulin-binding heat shock protein. Processes involved in redox signaling (Figure 4.5) were significantly affected as genes annotated to encode dehydroascorbate reductase, glutathione peroxidase and superoxide dismutase were down-regulated. However, genes annotated to encode members of the family of glutaredoxin proteins were up-regulated. Glutaredoxins are able to revert oxidative modifications on target enzymes and catalyse both S-thiolation and dethiolation (Holtgrefe *et al.*, 2008; Lind *et al.*, 1998; Shelton *et al.*, 2005). Cytosolic GAPDH was among the proteins discovered as potential targets of thioredoxins in plants (Wong *et al.*, 2004; Yamazaki *et al.*, 2004).

Hormone metabolism

Hormone metabolism (Figure 4.5) showed decreases in genes for enzymes of jasmonate and auxin biosynthesis, of particular note was the down-regulation of IAR3 (indole-3-acetic acid (IAA)-Ala-resistant), which encodes an auxin conjugate hydrolase. Brassinosteroids have been shown to reduce free IAA levels in soybean epicotyls (Zurek and Clouse, 1994) and repress IAR3 expression (Davies *et al.*, 1999). Therefore, the repression might indicate the possible involvement of brassinosteroids in the lumichrome mediated growth promotion. Increases in genes for ABA synthesis and a strong induction of genes annotated to encode ethylene biosynthesis enzymes were observed.

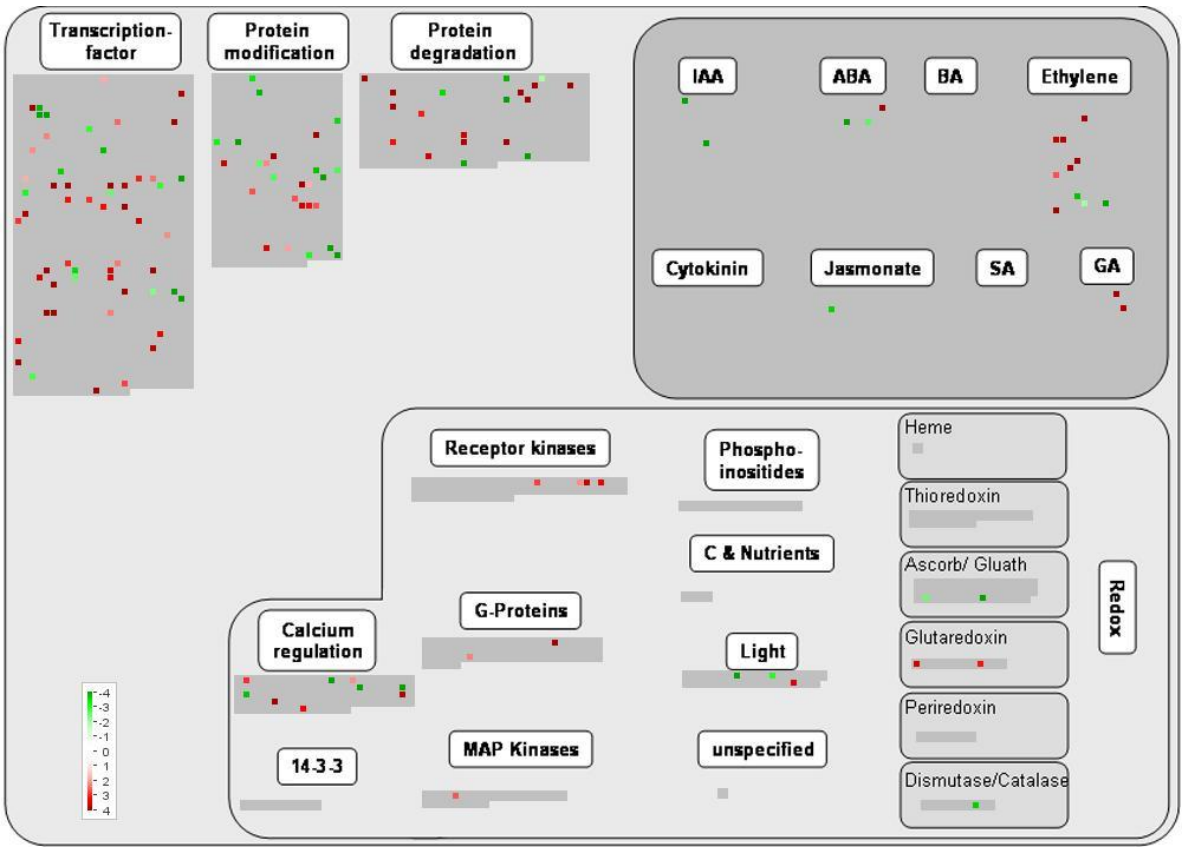


Figure 4.5 MapMan visualization of genes annotated to regulatory processes that showed differential expression P = (<0.05). Red represents genes that are induced, green represents genes that are repressed. The magnitude of change is indicated in the color scale represented in the figure (between +5 and -5).

A strong increase in the expression of the gene for aldehyde oxidase was observed. Aldehyde oxidase, which is a molybdenum associated enzyme, catalyses the last step of ABA synthesis where abscisic aldehyde is oxidized to abscisic acid and may control ABA accumulation after water stress (Walker-Simmons *et al.,* 1989). With regards to ethylene synthesis – genes annotated to encode ACC synthase and ACC oxidase were induced. These gene products play an important role in the ethylene biosynthetic pathway and up-regulation indicates an increase in ethylene synthesis. The gene for the ethylene response factor 1 (ERF1) was up-regulated which encodes a positive regulator that is necessary and sufficient for the activation of known responses mediated by the ethylene pathway (Chao *et al.*, 1997). The gene for the ethylene receptor 2 (LeETR2) was down-regulated. Ethylene is known to play a role in regulating root hair formation as well as the growth of primary, lateral and adventitious roots (Dolan, 1997). As lumichrome elicits growth in roots, increases in the levels of ethylene seem to play an important role. Moreover, both nitrogen deficiency and overdose can increase ethylene production (Tari and Bowen, 1995). Interestingly, the ethylene signaling pathway is negatively regulated by ethylene itself through ethylene receptors (Alonso and Stepanov, 2004) and the treatment with brassinosteriods has been shown to stimulate ethylene production, potentially via the regulation of genes involved in ethylene synthesis (Arteca and Arteca, 2001; Yi *et al.,* 1999). Increases in sterol synthesis, ethylene biosynthesis and reduced expression of IAR3 might indicate brassinosteroids to be a strong candidate for the involvement in the growth promotion. Genes encoding proteins involved in gibberellin synthesis and degradation showed very strong induction, especially the oxidoreductase (2OG-Fe(II) oxygenase family protein) and the gibberellin 2-oxidase, responsible for the inactivation of active gibberellins (Oh *et al.,* 2006).

Protein degradation, modification and signaling processes

Genes for protein degradation (Figure 4.5) showed strong differential gene expression, with particular note to cysteine and aspartic proteinases being highly induced. In response to environmental abiotic and biotic factors, cellular proteins have to be rebuilt. Misfolded and damaged proteins are eliminated by protein degradation and replaced by newly formed proteins, i.e. cold and heat shock proteins (see above), dehydration-induced proteins and pathogenesis-related proteins (Grudkowska and Zagdańska, 2004). Moreover, cysteine proteases have been shown to be involved in different aspects of plant defence (Estelle, 2001) and they are induced during various plant stresses (Kinoshita *et al.,* 1999). In tomato specifically, an aspartic proteinase has been suggested to take part in the hydrolysis of extracellular pathogenesis-related proteins (Rodrigo *et al.,* 1991). Thus, the increase in gene

expression for proteinases again point to increases in abiotic and biotic stress responses induced by lumichrome. Additionally, this might be the explanation as to why there is such a strong increase in heat shock induced gene expression in this study. Most of the genes that function at the level of post-translational modification (Figure 4.5) and displayed differential expression were repressed. An ankyrin-kinase and SYM10 displayed the highest level of repression (Figure 4.5). There were marked increases in transcript levels of genes that encode phosphatases 2C (PP2C), tyrosine phosphatase, serine/threonine kinase and the galactosyltransferase family. Tyrosine phosphatases are involved in major signaling networks in plants. In addition, tyrosine phosphatases regulate the activity of mitogen-activated protein kinases (MAPKs) and there have been studies linking H_2O_2, kinases and phosphatases (Hancock *et al.*, 2006). Liu *et al.* (2008) investigated the mechanism of nitrate-induced lateral root formation through microarray analysis. The reception and transduction of NO_3^- involved important protein kinases e.g. serine /threonine kinases and transcription factors e.g. a F-box protein. Genes annotated to encode receptor kinases, G-proteins and MAP kinases were induced (Figure 4.5).

Striking changes were found for genes encoding proteins assigned to calcium signaling (Figure 4.5). Among genes induced upon lumichrome treatment, the gene encoding calmodulin (CaM) showed the strongest induction. CaM sense nanomolar changes in Ca^{2+} concentrations and act as molecular switches to regulate other proteins and enzymes. CaM-binding proteins are thought to be the response elements through which the Ca^{2+}/CaM second messenger system effects signal transduction (Sotirios *et al.*, 2006). Increases in gene expression of genes encoding CaMs might be an indication of increases in signal transduction processes that are triggered by lumichrome. The gene annotated to a CaM -binding heat shock protein showed the highest suppression. Heat shock proteins that bind to calmodulin have been implicated to play a role in various processes i.e. functioning as chaperones to stabilize calmodulin (Nepomuceno *et al.*, 2002) and the involvement of this gene with ABA stress response reactions (Bressan, 1998).

Signaling gene products involved in light (Figure 4.5) processes showed a decrease in gene expression, specifically a putative *SCARECROW* gene regulator and the photolyase/blue-light receptor (PHR2). In *Arabidopsis thaliana*, *SCARECROW* (*SCR*) is essential for the asymmetric division of the cortex/endodermis progenitor cell in the root (Di Laurenzio *et al.*, 1996). The occurance of multiple and diverse signaling networks as result of lumichrome

treatment is a testament to the complex mode of downstream events triggered by lumichrome in regulating plant growth and performance.

PROTEIN PROFILING

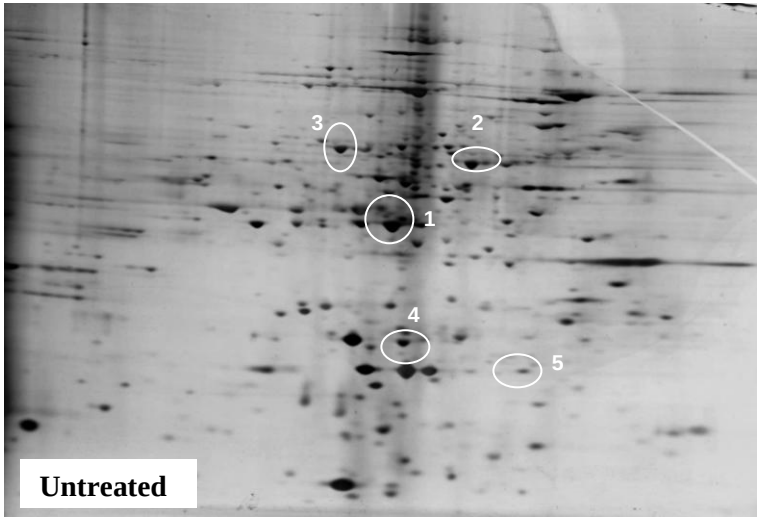
Proteomic analysis of lumichrome-induced changes in tomato roots

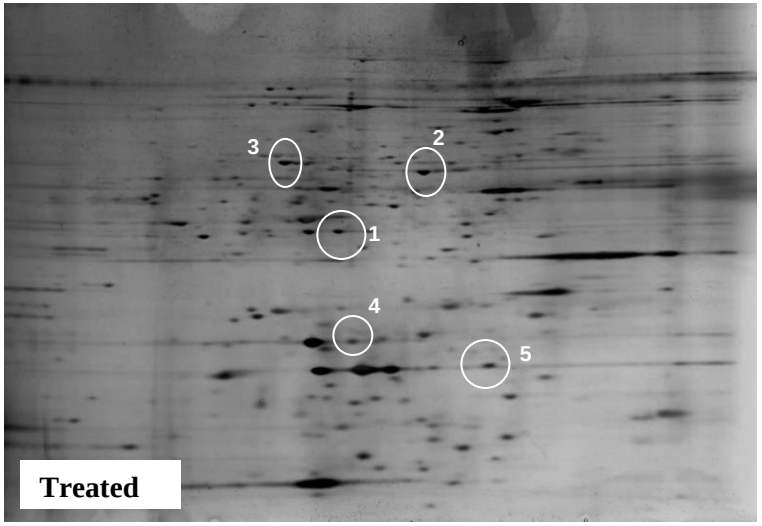
2D-PAGE of lumichrome treated and untreated roots resulted in ten spots displaying differential protein expression, which were excised and subjected to trypsin digest. The samples were analysed with Nano-LC-MS/MS and confirmed with MALDI-TOF-MS. Protein identification was based on raw MS/MS data using the Mascot (www.matrixscience.com) search engine. From the five proteins that could be identified (Table 1; Figure 4.6), only one protein was up-regulated in lumichrome treated roots and displayed protein levels corresponding to the transcriptomic data. This protein was identified as Chaperonin 21. Chaperonins are required for correct folding and are induced under stress conditions. Spot number 3, identified as glycine hydroxymethyltransferase, was down-regulated in lumichrome treated roots on the protein level, but did not show differential expression on the RNA level. This enzyme catalyses the reversible conversion of serine and tetrahydrofolate (THF) to glycine and N_5,N_{10}-methylene tetrahydrofolate. Interestingly, three enzymes involved in glycolysis, triose phosphate isomerase (TPI), glyceraldehyde-3-phosphate dehydrogenase (GAPDH, cytosolic, phosphorylating NAD specific) and phosphopyruvate hydratase or enolase were identified as being less abundantly present after lumichrome treatment. Proteomic data corroborated the transcriptomic data displayed for enolase showing significantly lower levels in lumichrome treated roots. However, GAPDH and TPI showed no significant difference in transcript levels of lumichrome treated roots compared to the untreated controls. GAPDH was analyzed in detail to determine its importance in the lumichrome mediated growth promotion.

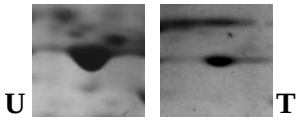
Table 1 Identification of significantly differentially expressed proteins in response to lumichrome in tomato roots, confirmed by CapLC-ESI-MS/MS and MALDI-MS analysis. Spot numbers correspond to those indicated in Figure 4.6

Spot number	***Up-regulation/ down-regulation***	***Mr/pI****	***Protein name***	***MOWSE Score*****	***Nr. Matched peptides******
1	down-regulated	28843/ 5.70	glyceraldehyde-3-phosphate dehydrogenase (cytosolic)	131/49	11
2	down-regulated	47768/ 5.68	phosphopyruvate hydratase (enolase)	41/49	15
3	down-regulated	51764/ 7.12	glycine hydroxymethyltransferase	76/48	13
4	down-regulated	27023/ 5.73	triose phosphate isomerase (cytosolic)	153/49	13
5	up-regulated	26546/ 6.85	chaperonin 21 precursor	97/50	14

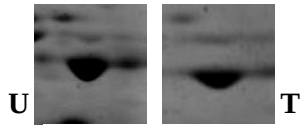
* Molecular mass and isoelectric point of the protein match. ** MOWSE = molecular weight search, MOWSE score – statistical probability of true positive identification of predicted proteins. *** Number of peptides matching predicted protein sequences







Spot number 1



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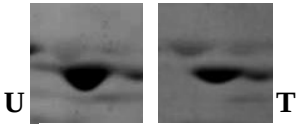

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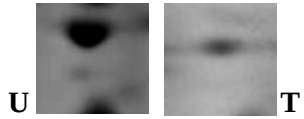

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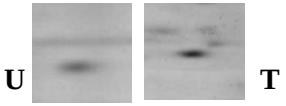

Spot number 5

Figure 4.6 Representative 2-DE gels of total soluble proteins from untreated and treated root proteins of tomato. Proteins on the gels were silver stained. Individual spot numbers 1-5 indicate the specific spot in the untreated (U) and treated (T) gels. Spot number 1 – 5 denote the identifications detailed in Table 1.

METABOLITE PROFILING

Minor metabolic changes induced upon lumichrome treatment in tomato roots

A total of 78 metabolites were measured in tomato roots and significant differences between samples assessed by performing the Student *t*-test ($P < 0.05$) embedded in the Microsoft Excel software (Microsoft, Seattle). Considering all 78 metabolites, the principal component analysis resulted in no clear groupings or recognizable patterns between untreated and treated roots. Between treated and untreated roots, only three metabolites displayed significant differences in metabolite levels (Supplementary data, Table B). Glutamine and pyroglutamic acid levels were significantly increased whilst only galactonic acid-1,4 lactone levels were decreased in lumichrome treated roots. Galactonic acid-1,4 lactone is a precursor for ascorbic acid biosynthesis, which is an important anti-oxidant involved in a range of stress-related processes (Conklin and Barth, 2004). In concurrence with this, the microarray analysis showed that processes involved in redox signaling were significantly affected as genes annotated to dehydroascorbate reductase, glutathione peroxidase and superoxide dismutase were down-regulated. However, lumichrome treated plants seem to exhibit signs of stress and one would think that enzymes involved in redox processes would be up-regulated as reactive oxygen species are normally generated when plants experience some forms of abiotic or biotic stress. Furthermore, as the down-regulation of the identified four proteins (see section: Protein Profiling) in the proteomic analysis was not reflected in the metabolite levels, it is suggested that post-translational modification of primary metabolism might play a role in maintaining steady state metabolite levels.

TARGETED ANALYSIS OF GAPDH

As the proteomic results revealed that glyceraldehyde-3-phosphate dehydrogenase (GAPDH; phosphorylating NAD-specific) was down-regulated in tomato roots, northern blot analysis was completed to investigate if the gene expression levels also reflected the proteomic results and to verify the importance of GAPDH in the lumichrome induced growth promotion. Untreated control and lumichrome treated tomato roots were subjected to northern blot analysis to investigate the levels of mRNA coding for GAPDH. As shown in Figure 4.7 A the expression levels of GAPDH transcripts in lumichrome treated roots were slightly higher than in the untreated control plants.

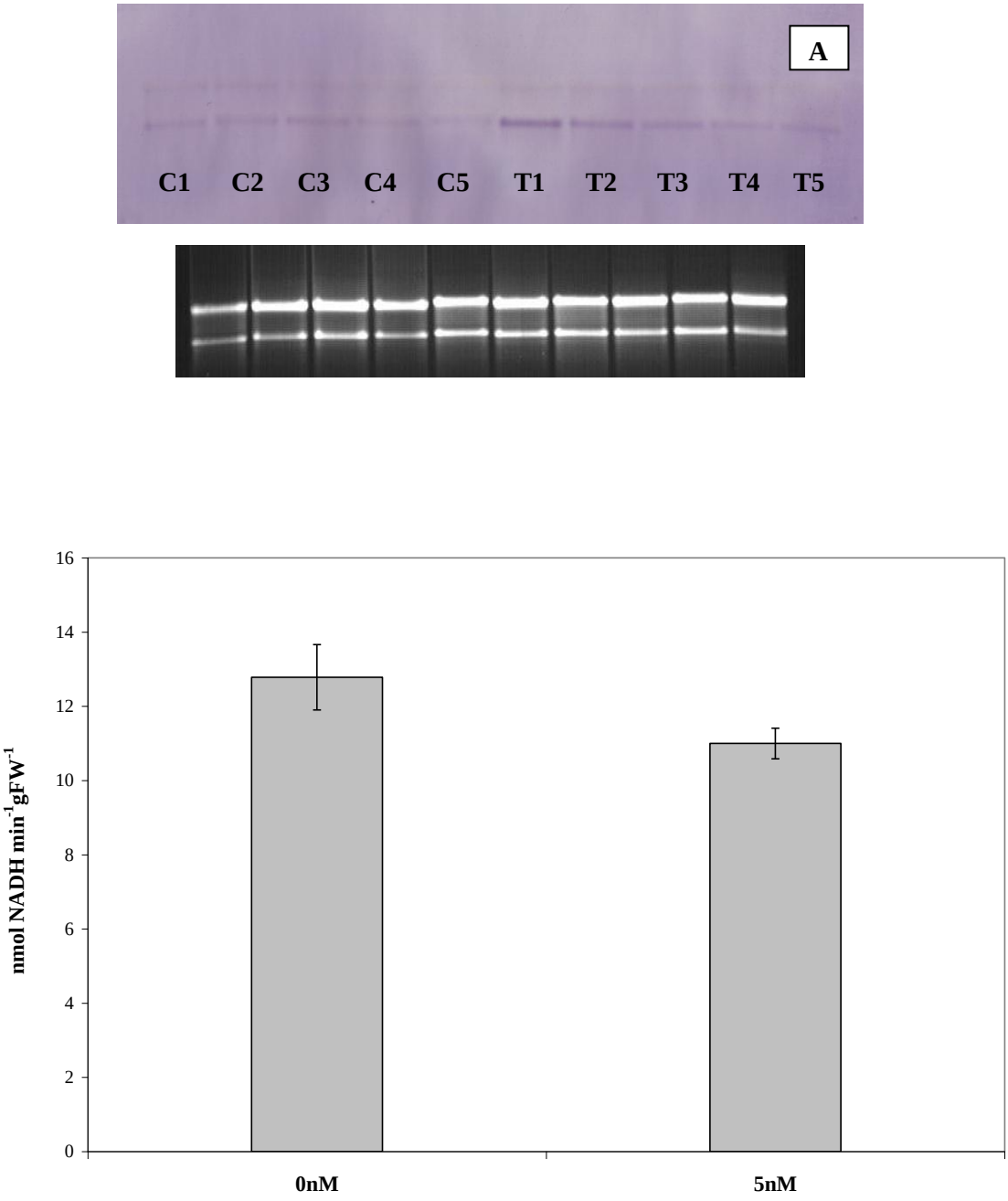

Figure 4.7 Targeted analysis of the effects of lumichrome on glyceraldehyde-3-phoshate dehydrogenase (GAPDH) in untreated control and lumichrome treated tomato roots. **A)** Northern blot analysis (top) of transcripts in treated plants. The gel (bottom) represents the ethidium bromide stained ribosomal subunits that were used to verify equal loading. Lane C1 – C5: untreated control roots; Lane T1 –T5: lumichrome treated roots. **B)** Enzyme activities of treated (5 nM) and untreated (0 nM) roots. Values calculated as mean ± SE of three determinations; n = 5 and significance tested by Student *t*-test ($P < 0.05$).

In addition, enzyme activity determinations for GAPDH were undertaken and no significant differences were found between lumichrome treated roots and untreated controls (Figure 4.7 B). As with *Lotus japonicus*, the protein levels did not parallel those of the RNA suggesting that GAPDH might be under post-translational regulation. GAPDH has been previously shown to be subjected to translational and multiple post translational control, especially when subjected to stress (Chang *et al.*, 2000; Laukens *et al.*, 2001; Bustos and Iglesias, 2003). Additionally, one might speculate that the apparent lower abundance of three glycolytic enzymes in protein gels after lumichrome treatment might be due to complex formation or posttranslational modification leading to a different migration pattern of the three proteins in the 2D-gels.

Differentially expressed genes in tomato and *Lotus japonicus* in response to lumichrome treatment

In chapter 3, a similar study was undertaken with another plant species *Lotus japonicus*. In order to narrow down candidate genes for further investigation, we compared the two transcript profiles of *Lotus* and tomato to investigate whether orthologous genes respond in a similar manner to lumichrome treatment between *Lotus* and tomato. Six genes, which were concurrently expressed in tomato and *Lotus*, were identified (Table 2).

Table 2 Common differentially expressed genes between tomato and *Lotus japonicus* in response to lumichrome

GENE	***BIOLOGICAL FUNCTION***	***UP-REGULATED / DOWN-REGULATED***
WRKY 6	Transcription factor family	Up-regulated
UDP-glucoronosyl/UDP-glucosyl transferase family protein	Large enzyme families (UDP glucosyl and glucoronyl transferases)	Up-regulated
ACC synthase	Hormone metabolism - ethylene	Up-regulated
DAHP synthase	Secondary metabolism	Up-regulated
Laccase	Cell wall metabolism	Up-regulated
Ca^{2+}-ATPase	Transport	Down-regulated

WRKY 6 was identified as a member of the WRKY transcription factor family that was up-regulated in response to lumichrome in both *Lotus* and tomato. WRKY6 is involved in controlling processes related to pathogen defence, wound response and senescence (Eulgem *et al.*, 2000; Robatzek and Somssich, 2001; Robatzek and Somssich, 2002). In *Arabidopsis*, WRKY6 increase pathogen-related 1 (*PR1)* promotor activity and a receptor-like kinase, *SIRK*, (Robatzek and Somssich, 2002) while WRKY6 also up-regulate *AtTRXh5*, a member of the cytosolic thioredoxin *h* family (Laloi *et al.*, 2004).

Transcript levels of an UDP-glucoronosyl/UDP-glucosyl transferase family protein were up-regulated, which is consistent with previous studies showing that wounding and pathogens induced the transcription of glucosyltransferase genes. For example the *Twi1* gene, which is a glucosyltransferase homologue, is rapidly induced in tomato plants in response to both wounding and pathogen attacks (O'Donnell *et al.*, 1998). Again, this points to increased disease resistance and the induction of defence related genes upon lumichrome treatment.
Regarding plant hormones, the gene that showed differential expression in both tomato and *Lotus* corresponded to the gene annotated to ACC synthase, the enzyme that catalyzes the committed step in the biosynthesis of ethylene. Enhanced ethylene production is an early, active response of a plants' perception of pathogen attack and is associated with the induction of defence reactions (Boller, 1991; van Loon *et al.*, 2006; Morgan and Drew, 1997). Lumichrome appears to trigger similar hormone responses observed during plant-pathogen interactions (Navarro *et al.*, 2008; Grant and Jones, 2009).

DAHP synthase, the first enzyme of the shikimate pathway, was induced in both *Lotus* and tomato roots. Shikimate is a precursor to several important signaling compounds, including the aromatic amino acids used for auxin, catecholamine, alkaloid and flavonoid biosynthesis. As with the previously mentioned genes, this gene has been implicated in plant defence responses (Keith *et al.*, 1991; Taguchi *et al.*, 1998).

Furthermore, plant laccases are involved in phenolic/flavonoid metabolism and lignin biosynthesis, which are considered to play indirect roles in defence against pathogens (Wang *et al.*, 2008). Gene expression levels of laccase were up-regulated in response to lumichrome. The gene annotated to Ca^{2+}-ATPase displayed lower levels of gene expression in lumichrome treated tomato and *Lotus* roots. Nemchinov *et al.* (2008) suggested that calcium acts not only as an important second messenger in the activation of resistance responses but is possibly also

a downstream mediator of the inhibition of the spread of invading pathogens and the completion of defence reactions. Accordingly, they suggested that the existing model of hypersensitive response (HR) should include the Ca^{2+}-ATPase as an important component of the HR to pathogens in plants.

The fact that lumichrome treatment resulted in altered gene expression levels not confined to a single pathway suggests that signal transduction is triggered in a distinct and unique manner. Therefore, we conclude that the six common genes that were differentially expressed in both lumichrome treated *Lotus* and tomato, in general point to plant defence responses to pathogens. These genes will be investigated extensively in the future to unravel the complex novel network on lumichrome induced growth promotion.

CONCLUSION

In summary, we have shown that lumichrome elicits growth at nanomolar concentrations in tomato roots. The treatment with lumichrome caused complex changes in gene expression, affecting mostly genes associated with RNA regulation of transcription, protein synthesis/degradation/modification and stress and defence. Interestingly, three proteins involved in glycolysis were down-regulated. Low correlations between changes in transcript levels and the effects of lumichrome treatment on protein and metabolite levels were observed. GAPDH was investigated in more detail and seem to be under post-translational modification. Lumichrome seems to induce similar changes in the gene expression of six orthologous genes in both lumichrome treated *Lotus* and tomato. These genes all relate to defence and pathogen related responses. These genes will be targeted for further analysis. Lumichrome possibly mimicks a pathogen attack and therefore might trigger changes in hormone balances which often occur during plant-pathogen interactions (Navarro *et al.*, 2008; Grant and Jones, 2009).

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SUPPLEMENTARY DATA

DNA MICROARRAY ANALYSIS

Table A Differentially expressed genes in tomato roots in response to lumichrome *****Mean ratio of the normalized data between lumichrome treated and control plants.

METABOLISM OVERVIEW

Up/Down Regulated	Annotation	Biological Function	Gene ID	Value*
Up-regulated	folylpolyglutamate synthase (fpgs2)	*C1- metabolism*	7.4.3.5	3.69
Up-regulated	Adenylate kinase (ATP-AMP transphosphorylase)	*Nucleotide metabolism*	1.1.9.6	4.05
Up-regulated	guanylate kinase		5.1.8.5	3.97
Down-regulated	AMP deaminase -related		5.1.17.4	-3.71
Up-regulated	Histidine decarboxylase (HDC) (TOM92)	*Amino acid metabolism – degradation*	4.4.19.4	6.00
Up-regulated	alanine:glyoxylate aminotransferase 2 homolog		1.3.2.14	2.03
Downregulated	delta-1-pyrroline-5-carboxylate dehydrogenase precursor		5.3.4.11	-4.26
Up-regulated	tryptophan synthase	*Amino acid metabolism – synthesis*	2.2.8.12	3.69
Up-regulated	Phospho-2-dehydro-3-deoxyheptonate aldolase 2		8.3.12.7	4.53
Up-regulated	Glutamate decarboxylase (GAD) (ERT D1)		4.1.17.13	6.47
Down-regulated	tryptophan synthase, beta subunit, putative		8.4.14.5	-4.03
Down-regulated	phosphoribosylanthranilate transferase		4.1.4.1	-3.96

Down-regulated	prephenate dehydratase		6.4.14.20	-3.44
Down-regulated	S-adenosylmethionine synthetase 2 (Methionine adenosyltransferase 2)		6.3.1.18	-4.03
Down-regulated	delta-1-pyrroline-5-carboxylate dehydrogenase precursor		5.3.4.11	-4.26
Down-regulated	prephenate dehydratase		6.4.14.20	-3.44
Down-regulated	dehydroquinate synthase		5.2.4.21	-6.16
Down-regulated	cytosolic cysteine synthase		8.4.11.3	-5.56
Up-regulated	orcinol O-methyltransferase	*Secondary metabolism*	8.3.17.21	2.79
Up-regulated	laccase		2.1.2.11	5.93
Up-regulated	Phytoene synthase 1		2.3.17.16	3.95
Up-regulated	Geranylgeranyl pyrophosphate synthetase		6.2.19.7	2.33
Up-regulated	mevalonate kinase		4.1.1.12	8.14
Up-regulated	Phytoene synthase 1		2.3.17.16	3.95
Up-regulated	Phytoene dehydrogenase		6.1.1.2	5.28
Up-regulated	putative flavonol synthase		4.1.16.11	2.68
Down-regulated	Polyphenol oxidase F, (Catechol oxidase)		1.4.13.1	-2.41
Down-regulated	geranylgeranyl reductase		2.2.7.2	-2.43
Up-regulated	steroid 5alpha-reductase	*Lipid metabolism*	2.1.5.6	3.75
Up-regulated	1-acyl-sn-glycerol-3-phosphate acyltransferase		8.1.14.1	3.54
Down-regulated	putative sphingolipid delta 4 desaturase DES-1		5.1.1.10	-1.82
Down-regulated	omega-6 fatty acid desaturase		8.3.3.15	-4.21
Down-regulated	Acyl-[acyl-carrier protein] desaturase		8.1.11.16	-1.84
Down-regulated	Acyl-CoA-binding protein (ACBP)		5.2.2.2	-2.56
Up-regulated	xyloglucan endotransglucosylase-hydrolase XTH5	*Cell wall modification*	6.2.17.21	3.13
Down-regulated	xyloglucan endotransglucosylase-hydrolase XTH7		3.3.2.2	-2.29
Up-regulated	Brassinosteroid-regulated protein BRU1 precursor		3.3.17.16	5.65

Up-regulated	Pectinesterase 3 precursor (Pectin methylesterase 3)	*Cell wall pectin*	1.2.17.13	6.18
Up-regulated	pectinesterase family		6.1.19.11	2.20
Up-regulated	Pectinesterase 1 precursor (Pectin methylesterase 1)		3.3.17.12	3.47
Down-regulated	pectinesterase family		4.3.3.18	-4.51
Down-regulated	cellulose synthase family protein	*Cell wall – cellulose synthesis*	7.3.10.16	-6.14
Up-regulated	xyloglucan endotransglucosylase-hydrolase XTH9	*Cell wall – degradation*	7.4.14.19	2.66
Up-regulated	polygalacturonase		1.2.12.1	3.11
Up-regulated	pectate lyase		8.2.17.14	3.71
Down-regulated	glycosyl hydrolase family 5/cellulase		6.1.6.5	-1.73
Down-regulated	putative UDP-glucose dehydrogenase 2	*Cell wall – precursor synthesis*	6.4.6.7	-2.97
Up-regulated	callose synthase	*Minor CHO metabolism*	7.3.20.4	4.53
Up-regulated	citrate synthase -related protein	*Gluconeogenese/ glyoxylate cycle*	3.3.20.12	3.54
Down-regulated	aldehyde dehydrogenase 1 precursor	*Fermentation*	5.1.6.18	-4.68
Up-regulated	phosphoenolpyruvate carboxylase 2	*Glycolysis*	1.1.4.2	3.68
Down-regulated	Enolase (2-phosphoglycerate dehydratase)		7.3.2.19	-3.74
Up-regulated	ADP-glucose pyrophosphorylase l	*Major CHO metabolism – starch synthesis*	1.3.3.5	6.66
Down-regulated	glucose-1-phosphate adenylyltransferase		5.3.16.8	-4.25
Down-regulated	alpha glucosidase II		6.4.17.21	-4.02
Down-regulated	starch synthase II-2 precursor		1.4.8.13	-4.11
Up-regulated	Vacuolar invertase	*Major CHO metabolism – sucrose degradation*	2.2.17.20	3.15
Up-regulated	NADH glutamate synthase isoform 1	*N-metabolism*	4.4.12.2	6.21
Up-regulated	cytochrome c oxidase subunit Vb -related	*Mitochondrial electron transport / ATP synthesis*	5.4.17.19	2.92
Down-regulated	putative cytochrome c oxidase subunit VIa precursor	*mitochondrial electron transport / ATP synthesis*	2.3.14.18	-2.93

CELLULAR RESPONSE

Up/Down Regulated	Annotation	Biological Function	Gene ID	Value
Up-regulated	resistance protein RGC2	*Biotic stress*	8.4.19.5	3.83
Up-regulated	tospovirus resistance protein B		5.1.3.3	5.51
Up-regulated	disease resistance protein		3.2.17.12	5.23
Up-regulated	Pathogenesis-related leaf protein 4 precursor (P4)		3.2.8.18	5.44
Down-regulated	PR5-like protein		6.3.1.19	-3.49
Down-regulated	disease resistance protein BS2		4.3.11.10	-5.42
Up-regulated	heat shock transcription factor family	*Abiotic stress – heat*	1.2.17.15	5.22
Up-regulated	heat shock transcription factor 5 (HSF5)		4.2.18.19	5.88
Up-regulated	hypothetical protein		3.2.15.21	5.66
Up-regulated	Heat shock protein 83		8.1.19.14	3.27
Up-regulated	DnaJ protein family		2.2.19.14	2.38
Down-regulated	calmodulin-binding heat-shock protein		4.1.3.8	-5.55
Up-regulated	putative transcriptional activator CBF1	*Abiotic stress – drought/salt*	1.1.16.11	1.87
Down-regulated	fiber protein Fb2		7.4.2.17	-2.44
Down-regulated	late-embryogenesis protein homolog	*Abiotic stress – unspecified*	5.3.4.1	-3.52
Down-regulated	germin-like protein, putative		6.3.2.15	-4.51
Up-regulated	glutaredoxin family protein	*Redox - glutaredoxin*	2.4.19.4	3.56
Down-regulated	Probable phospholipid hydroperoxide glutathione peroxidase	*Redox – ascorbate/glutathione*	1.3.3.2	-4.43
Down-regulated	dehydroascorbate reductase		6.2.4.15	-2.46
Down-regulated	Superoxide dismutase [Cu-Zn] superoxide dismutase	*Redox – dismutases/catalases*	6.4.1.13	-3.62

Up-regulated	structural maintenance of chromosomes 1 protein	*Cell division*	8.2.12.3	1.71
Down-regulated	Ribosome recycling factor (RRF)		1.2.1.17	-4.02
Up-regulated	CDK-activating kinase	*Cell cycle*	4.2.12.6	2.22
Up-regulated	cyclophylin -related protein		1.3.17.8	3.65
Up-regulated	Peptidyl-prolyl cis-trans isomerase (PPIase)		3.1.7.6	6.37
Up-regulated	YABBY-like transcription factor GRAMINIFOLIA	*Development*	4.1.11.4	4.41
Up-regulated	Floral homeotic protein AGAMOUS (TAG1)		5.1.16.2	3.17
Up-regulated	late embryogenesis (Lea)-like protein ER5, ethylene-responsive		5.4.16.20	3.51
Up-regulated	TAGL1 transcription factor		8.4.19.12	3.18
Up-regulated	No apical meristem (NAM) protein family		8.1.20.1	2.19
Down-regulated	TPA: putative phytosulfokine peptide precursor		3.1.15.20	-1.91
Down-regulated	late-embryogenesis protein homolog		5.3.4.1	-3.52
Down-regulated	NIN-like protein 2		5.1.11.15	-4.45
Down-regulated	pepper MADS-box protein		2.4.1.15	-2.51
Down-regulated	Notchless protein homolog		1.1.4.10	-3.60
Down-regulated	MADS-box transcription factor FBP29		1.1.8.20	-3.09

LARGE ENZYME FAMILIES

Up/Down Regulated	Annotation	Biological Function	Gene ID	Value
Up-regulated	Cytochrome P450	*Cytochrome P450*	7.1.11.2	5.44
Up-regulated	putative flavonoid 3',5'-hydroxylase		1.4.2.10	3.78
Up-regulated	Cytochrome P450		2.1.20.2	3.70
Down-regulated	glycosyl hydrolase family 5/cellulase ((1-4)-beta-mannan endohydrolase)	*Gluco-, galacto- and mannosidases*	6.1.6.5	-1.73
Down-regulated	alpha glucosidase II		6.4.17.21	-4.02
Up-regulated	putative peroxidase	*Peroxidases*	7.4.12.2	4.08
Down-regulated	peroxidase, putative		3.2.5.6	-5.58
Up-regulated	probable glucosyltransferase	*UDP glucosyl and glucoronyl transferases*	2.1.18.6	2.53
Up-regulated	exostosin family protein		8.2.19.14	3.57
Up-regulated	cold-induced glucosyl transferase		3.1.18.6	2.63
Down-regulated	glycosyltransferase family 1		8.1.2.16	-4.13
Down-regulated	glucuronosyl transferase homolog, ripening-related		8.1.7.18	-2.24
Down-regulated	glycosyl hydrolase family 17, putative beta-1,3-glucanase	*beta 1,3 glucan hydrolases*	8.4.16.3	-4.51
Up-regulated	putative lipase	*GDSL-motif lipase*	6.4.17.11	5.15
Up-regulated	GDSL-motif lipase/hydrolase protein		4.3.17.13	3.26
Down-regulated	probable glutathione transferase	*Glutathione S transferases*	1.3.6.4	-4.09
Down-regulated	short-chain dehydrogenase/reductase family protein	*Nitrilases*	7.3.7.18	-3.57

REGULATION OVERVIEW

Up/Down Regulated	Annotation	Biological Function	Gene ID	Value
		HORMONE METABOLISM		
Down-regulated	IAA-Ala hydrolase (IAR3)	*Auxin*	1.4.9.18	-4.12
Down-regulated	tuberisation-related protein		8.4.4.3	-6.06
Up-regulated	aldehyde oxidase	*Abscisic acid*	3.2.8.6	4.11
Down-regulated	Abscisic acid and environmental stress inducible protein TAS14 (Dehydrin TAS14)		6.4.1.15	-4.41
Down-regulated	Abscisic stress ripening protein		7.3.2.20	-2.66
Up-regulated	1-aminocyclopropane-1-carboxylate oxidase homolog (Protein E8)	*Ethylene*	6.4.3.12	3.72
Up-regulated	ethylene receptor homolog		4.2.18.6	2.68
Up-regulated	ethylene-forming-enzyme-like dioxygenase		7.1.2.19	3.81
Up-regulated	Pathogenesis-Related genes transcriptional activator PTI5		3.3.19.19	6.14
Up-regulated	ethylene response factor 1		3.1.3.11	4.72
Up-regulated	1-aminocyclopropane-1-carboxylate synthase 4 (ACC synthase 4)		4.2.17.17	4.32
Down-regulated	Ethylene receptor 2 (LeETR2)		8.4.2.21	-3.72
Down-regulated	multiprotein bridging factor 1		4.1.4.16	-1.95
Up-regulated	oxidoreductase, 2OG-Fe(II) oxygenase family protein	*Gibberellic acid*	3.2.5.9	7.97
Up-regulated	gibberellin 2-oxidase 2		8.4.17.20	3.94
Down-regulated	lipoxygenase (EC 1.13.11.12)	*Jasmonate*	8.2.6.1	-3.58

		SIGNALING		
Up-regulated	receptor-related protein kinase - like	*Receptor kinases*	4.4.14.20	3.42
Up-regulated	putative G protein coupled receptor	*G-proteins*	6.3.18.15	2.30
Up-regulated	mitogen-activated protein kinase kinase (MAPKK)	*MAP kinases*	2.2.18.6	2.65
Up-regulated	pheromone receptor-like protein	*Calcium*	1.2.17.11	2.95
Up-regulated	Calmodulin		6.3.17.11	5.24
Up-regulated	calcium-dependent protein kinase 3		2.2.18.9	2.15
Down-regulated	calcium-dependent protein kinasen		4.3.9.16	-3.75
Down-regulated	probable calcium-binding protein		2.2.1.7	-4.26
Down-regulated	calmodulin-binding heat-shock protein		4.1.3.8	-5.55
Down-regulated	potential calcium-transporting ATPase 9, plasma membrane-type		4.3.10.8	-4.39
Down-regulated	putative *SCARECROW* gene regulator	*Light*	2.2.11.8	-4.66
Down-regulated	photolyase/blue-light receptor (PHR2)		3.2.17.16	-3.02
		PROTEIN DEGRADATION		
Up-regulated	serine protease-like protein	*Subtilases*	2.2.17.21	4.14
Down-regulated	subtilisin-like proteinase		3.3.6.21	-3.51
Up-regulated	aspartic proteinase precursor	*Aspartate protease*	6.4.13.2	7.47
Up-regulated	ATP-dependent Clp protease ATP-binding subunit (ClpD)	*Serine protease*	8.1.19.16	3.08
Up-regulated	ubiquitin-specific protease 6 (UBP6)	*Ubiquitin – protease*	5.1.19.2	3.63
Up-regulated	putative E2, ubiquitin-conjugating enzyme UBC7	*Ubiquitin - E2*	5.1.11.2	3.64

Up-regulated	bg55	*Ubiquitin - E3*	3.1.11.4	3.17
Up-regulated	phloem-specific lectin PP2-like protein	*Ubiquitin - E3*	5.1.17.1	3.87
Up-regulated	putative F-Box protein	*Ubiquitin - E3*	1.2.16.17	6.83
Down-regulated	Proteasome subunit beta type 1 (20S proteasome alpha subunit F)	*Ubiquitin – proteasom*	7.4.3.16	-3.93
Down-regulated	26S protease regulatory subunit 6B homolog		3.2.12.21	-5.04
Up-regulated	cysteine protease	*Cysteine protease*	1.2.17.12	4.88
Up-regulated	cysteine proteinase		8.3.17.19	6.26
Down-regulated	cysteine protease		8.1.2.16	-4.13
Up-regulated	protease-related protein	*Protein degradation*	7.4.12.10	10.75
Down-regulated	peptidase family		4.1.6.12	-3.84

PROTEIN MODIFICATION

Up-regulated	protein phosphatase 2C (PP2C)	*Posttranslational modification*	4.2.10.14	3.65
Up-regulated	putative metallophosphatase		8.1.18.3	1.97
Up-regulated	protein tyrosine phosphatase		6.1.17.3	3.18
Up-regulated	galactosyltransferase family		6.1.17.17	3.59
Up-regulated	Serine/threonine Kinase		5.4.1.2	2.68
Up-regulated	protein kinase family		5.1.6.1	1.83
Up-regulated	receptor-like protein kinase		3.2.17.12	5.23
Down-regulated	MAP kinase phosphatise		3.2.18.3	-3.19
Down-regulated	ankyrin-kinase		3.2.19.14	-5.97
Down-regulated	leucine rich repeat protein kinase family		1.3.4.20	-3.81
Down-regulated	AtBgamma - like protein		1.1.16.10	-3.36

Down-regulated	calcium-dependent protein kinase		4.3.9.16	-3.75
Down-regulated	probable protein kinase		4.4.12.21	-2.74
Down-regulated	GAL83 protein		8.4.1.12	-4.29
Down-regulated	SYM10 protein		8.2.11.18	-5.41
Down-regulated	putative chaperonin containing TCP1		3.1.1.16	-3.49
Down-regulated	leucine rich repeat protein kinase family		1.3.4.20	-3.81
Down-regulated	MAP kinase phosphatase		3.2.18.3	-3.19

RNA REGULATION OF TRANSCRIPTION

Up/Down Regulated	Annotation	Biological Function	Gene ID	Value
Up-regulated	myb-related protein	*MYB domain transcription factor family*	6.1.16.19	2.91
Up-regulated	putative MYB transcription factor		2.3.16.10	3.93
Up-regulated	dehydration-induced myb-related protein Cpm7		8.4.19.16	3.62
Up-regulated	Floral homeotic protein AGAMOUS (TAG1)	*MADS box transcription factor family*	5.1.16.2	3.17
Up-regulated	MADS-box protein 15		5.4.16.16	2.94
Up-regulated	TAGL1 transcription factor		8.4.19.12	3.18
Down-regulated	MADS-box transcription factor FBP29		1.1.8.20	-3.09
Up-regulated	bHLH protein SPATULA (SPT)	*Basic Helix-Loop-Helix family*	1.4.7.1	5.72
Down-regulated	bHLH protein		7.1.4.13	-6.10
Up-regulated	heat shock transcription factor family	*Heat-shock transcription factor family*	1.2.17.15	5.22
Up-regulated	heat shock transcription factor 5 (HSF5)		4.2.18.19	5.88

Up-regulated	putative RING-H2 finger protein RHB1a	*C3H zinc finger family*	6.1.18.9	2.19
Up-regulated	putative homeodomain protein	*Homeobox transcription factor family*	6.3.16.12	1.82
Up-regulated	homeodomain leucine zipper protein HDZ2		8.4.16.2	4.75
Down-regulated	BEL1-related homeotic protein 11		4.4.1.4	-3.58
Down-regulated	homeobox 1 protein		8.3.1.9	-6.50
Up-regulated	transducin / WD-40 repeat protein family	*putative DNA-binding protein*	1.2.7.18	4.16
Up-regulated	proline-rich protein family		1.4.8.4	2.33
Up-regulated	WRKY family transcription factor	*WRKY domain transcription factor family*	8.1.11.1	2.99
Up-regulated	bZIP transcription factor BZI-2	*bZIP transcription factor family*	6.1.10.3	3.62
Up-regulated	ZIP DNA-binding protein		7.4.17.16	3.53
Up-regulated	bZIP DNA-binding protein HBF-1		4.1.17.17	3.50
Up-regulated	bZIP transcription factor		2.2.16.2	8.18
Down-regulated	transcription factor bZIP61 (BZIP61)		6.3.6.11	-2.55
Down-regulated	bZIP transcriptional activator RSG		3.2.20.7	-3.49
Down-regulated	transcription factor bZIP61 (BZIP61), pelota-related protein		6.3.6.11	-2.55
Down-regulated	NIN-like protein 2	*NIN-like bZIP-related family*	5.1.11.15	-4.45
Up-regulated	ZPT2-13	*C2H2 zinc finger family*	5.1.16.12	2.29
Up-regulated	zinc finger (C3HC4-type RING finger) protein family		8.4.19.9	4.41
Down-regulated	zinc finger protein		1.1.4.9	-2.92
Up-regulated	No apical meristem (NAM) protein family	*NAC domain transcription factor family*	8.1.20.1	2.19
Up-regulated	transcriptional co-activator (KELP) -related	*General Transcription*	2.1.6.14	5.71
Down-regulated	TFIIA		6.1.6.18	-2.29
Up-regulated	Transcriptional activator DEMETER (DNA glycosylase-related protein DME)	*Orphan family*	1.2.10.1	2.37
Up-regulated	YABBY-like transcription factor GRAMINIFOLIA	*C2C2(Zn) YABBY family*	4.1.11.4	4.41

Up-regulated	Pathogenesis-related genes transcriptional activator PTI6	*AP2/EREBP, APETALA2/Ethylene-responsive element binding protein family*	7.3.7.1	3.86
Up-regulated	putative transcriptional activator CBF1		1.1.16.11	1.87

TRANSPORT OVERVIEW

Up/Down Regulated	Annotation	Biological Function	Gene ID	Value
Up-regulated	plasma intrinsic protein 2,1	*Major Intrinsic Proteins – PIP*	8.2.11.16	2.03
Up-regulated	vacuolar ATPase subunit B	*P- and V-ATPases*	8.3.17.18	2.68
Down-regulated	Plasma membrane ATPase 1 (Proton pump 1)		1.1.12.9	-5.92
Down-regulated	Vacuolar ATP synthase subunit G 2		6.3.10.12	-3.25
Up-regulated	boron transporter	*Anions*	8.1.9.3	2.45
Up-regulated	amino acid transporter family	*Amino acids*	1.1.2.6	2.68
Down-regulated	Ca^{2+}-transporting ATPase-like protein	*Calcium*	4.3.10.8	-4.39
Down-regulated	potassium channel	*Potassium*	7.3.5.3	-3.75
Down-regulated	heavy-metal-associated domain-containing protein	*Miscellaneous*	7.3.3.20	-3.53
Down-regulated	MATE efflux protein - related		2.4.2.17	-5.37
Down-regulated	putative CMP-sialic acid transporter	*Transporter – sugars*	8.4.8.1	-4.98
Down-regulated	putative mitochondrial dicarboxylate carrier protein	*Metabolite transporters at the mitochondrial membrane*	6.1.19.2	-4.51

RNA-PROTEIN SYNTHESIS

Up/Down Regulated	Annotation	Biological Function	Gene ID	Value
Down-regulated	Eukaryotic translation initiation factor 3 subunit 9 (eIF-3 eta)	*Protein synthesis – initiation*	6.3.8.11	-11.03
Up-regulated	elongation factor Tu (EF-Tu), mitochondrial precursor	*Protein synthesis – elongation*	4.3.17.11	4.53
Up-regulated	ribosomal protein S26, cytosolic	*Protein synthesis – ribosomal protein*	4.1.9.1	5.24
Up-regulated	ribosomal protein S21 – like		2.1.3.2	3.60
Up-regulated	Ribosomal protein L34e		6.1.15.4	2.24
Down-regulated	60S ribosomal protein L4 (L1)		4.3.4.11	-3.49
Down-regulated	40S ribosomal protein S14 (RPS14B)		3.2.4.1	-3.64
Down-regulated	cytoplasmic ribosomal protein S14		1.4.4.20	-9.13
Down-regulated	40S ribosomal protein S20 (RPS20B)		1.1.9.5	-2.49
Down-regulated	40S Ribosomal protein S11		5.3.16.8	-4.25
Down-regulated	60S RIBOSOMAL PROTEIN L23A (L25)		1.3.2.7	-2.94
Up-regulated	isoleucine-tRNA ligase - like protein	*Amino acid activation*	1.1.19.2	5.73
Down-regulated	phenylalanyl-trna synthetase - like protein		5.2.6.6	-4.33

METABOLITE PROFILING

Table B Changes in metabolite abundance of tomato roots as a result of lumichrome treatment.

Up-regulated	***METABOLITE NAME***	***METABOLITE CLASS***	***RESPONSE RATIO* (5 nM/0 nM)***	***T-TEST*****
	Glutamine	Amino acids	2.00	0.02
	Pyroglutamic acid	Amino acids	1.53	0.02
Down-regulated	***METABOLITE NAME***	***METABOLITE CLASS***	***RESPONSE RATIO* (5 nM/0 nM)***	***T-TEST*****
	Galactonic acid-1,4-lactone	Polyhydroxy acids	0.84	0.05

* Response ratio = intensity of the mass of the specific metabolite normalized to fresh weight and the internal standard ribitol ** Significant changes were evaluated using t-test ($p \leq 0.05$)

CHAPTER 5

General Discussion and Conclusion

This study provides new insight into the molecular effects of the plant growth promoter lumichrome on the root metabolism of plants. The main aim of the work presented in this thesis was to investigate the molecular mechanism of the plant growth promoting substance lumichrome in the roots of the model plants *Lotus japonicus* and *Solanum lycopersicon* (tomato) through transcriptomic, proteomic and metabolomics analyses. The knowledge gained in these parallel analyses of both *Lotus japonicus* and tomato aided us in finding key genes involved in the growth stimulation. For the first time to our knowledge it was found that lumichrome seems to induce similar changes in the gene expression of six orthologous genes in both lumichrome treated *Lotus* and tomato. These genes all relate to defence and pathogen related responses.

As the effects of lumichrome as a plant growth promoter has not previously been tested on *Lotus japonicus* and tomato, basic growth studies were completed to determine if lumichrome indeed elicits plant growth at nanomolar concentrations, as proven in numerous previous studies (Phillips *et al.*, 1999; Matiru and Dakora, 2005a; Khan *et al.*, 2008). Both *Lotus japonicus* (Chapter 3) and tomato (Chapter 4) showed significant increases in root biomass when treated with 5 nM of lumichrome. Subsequently, to asses the impact of lumichrome on the root metabolism of *Lotus japonicus* and tomato and identify key genes involved in the growth stimulation, a comprehensive profile of differentially expressed genes, proteins and metabolites was compiled. Proteomics studies revealed that GS1 (*Lotus*) and GAPDH (*Lotus* and tomato) were present in lower abundance in lumichrome treated roots, therefore targeted analysis utilizing northern blots, western blots and the measurement of enzyme activities were completed to determine and verify their specific role in the lumichrome mediated growth promotion.

The treatment with lumichrome caused complex changes in gene expression. Generally, transcript profiling showed that the categories that were predominantly affected by

lumichrome in both *Lotus* and tomato, were genes associated with RNA regulation of transcription and signaling, protein synthesis/degradation/modification and stress and defence.

Lumichrome induced changes on the protein level resulted in the identification of five proteins in *Lotus* (Chapter 3; Table 1) and five proteins in tomato (Chapter 4; Table 1) being differentially expressed. From the ten proteins that were differentially expressed, only one protein was up-regulated whilst the majority of the differentially expressed proteins were down-regulated. After examining the functions of differentially expressed proteins, generally these results suggest that lumichrome seems to largely influence proteins involved in protein folding and down-regulate proteins involved in glycolysis. GAPDH was the one protein that was down-regulated in both lumichrome treated *Lotus* and tomato roots. Consequently, GAPDH was investigated in more detail in *Lotus* and tomato roots, along with GS1 in *Lotus* roots, to confirm their importance and relevance in lumichrome mediated growth promotion.

Therefore, we next turned our attention to the targeted analysis of GAPDH and GS1. Concerning GAPDH, northern blot analysis of both *Lotus* and tomato roots showed increased transcript levels in lumichrome treated plants. However, no significant difference was displayed in the microarray analysis. Moreover, no significant differences between the enzyme activities of lumichrome treated and untreated roots were found. Together these results indicate that GAPDH might be under post-translational control. The targeted analysis of GS1 was only completed for *Lotus*, which resulted in increased transcript levels in lumichrome treated roots. This was corroborated in the microarray analysis where increased transcript levels of GS1 were found in lumichrome treated roots. Conversely, no significant difference was measured in the enzyme activities and western blot analysis could not show differences on protein levels. One must be cautious in interpreting these results, as multiple isoenzymes exist for GS1 and the identified protein in the 2-DE gel might have been one of the isoenzymes displaying different migration behavior from the other isoenzymes.

The influence of lumichrome on the metabolome of *Lotus* roots was immense, however minute in tomato roots. From the 78 metabolites measured in *Lotus* roots, 46 metabolites displayed significant differences in their metabolite levels. Additionally, taking into account all 78 metabolites, the principal component analysis revealed that the metabolomes of lumichrome treated and untreated control roots were distinctly different and the analysis could separate them into two distinct groups of treated and untreated. Lumichrome induced changes with respect to metabolite classes in *Lotus* roots, showed that 46 % of metabolite changes

occurred in the amino acid pool where a general decrease was observed. Other changes in metabolite levels point to the possibility that lumichrome causes the plant to respond as if they were under nitrogen deficiency and oxidative stress. Unexpectedly and in contrast to *Lotus*, the metabolite analysis for tomato resulted in only three metabolites displaying significant differences in their levels. Generally, no correlation was observed between changes in metabolite levels and differential gene expression.

In order to postulate a possible mode of action of how lumichrome precisely promotes plant growth, candidate genes were narrowed down for further investigation. Transcript profiles of *Lotus* and tomato were compared to investigate whether orthologous genes can be identified that responded in a similar manner to lumichrome. Of great interest was the finding that six genes, all related to defence and pathogen responses, were concurrently expressed in both tomato and *Lotus* (Chapter 4; Table 2). These six genes play crucial roles as transcription factors, as UDP glucosyl and glucoronyl transferases, in hormone metabolism, in secondary metabolism, in cell wall metabolism and lastly in transport processes.

Plants have developed diverse defence systems to halt pathogen growth. These responses include physical changes (e.g. cell wall thickening and callose deposition) and biochemical responses (e.g. the production of ROS/signaling compounds such as salicylic acid, jasmonic acid, abscisic acid, ethylene) that perturb infection (Chisholm *et al.* 2006; Jones and Dangl 2006). Additionally, de novo production of various defence related proteins and secondary metabolites such as phytoalexins and various phenolics can accumulate both locally and systemically (Hammerschmidt 1999; van Loon *et al.* 2006; Bolton, 2009). Considering the above mentioned, lumichrome treated plants exhibited almost all of the above characteristics and parallels other responses of plants to pathogens.

One finds great similarities between the response of *Lotus* and tomato when treated with lumichrome and how plants react to for example biotrophic fungi. Necrotrophic fungi kill plant cells rapidly after infection to feed on dead tissue, however biotropic fungi acquire nutrients from living plant tissue (Doehlemann *et al.*, 2008). Recently, Doehlemann *et al.* (2008) investigated the interplay between the fungus *Ustilago maydis* and maize plants with confocal microscopy, global expression profiling and metabolic profiling. Early responses revealed the induction of defence mechanisms, changes in hormone signaling and the induction of antioxidant and the secondary metabolism. The basal plant defence machinery is induced upon the recognition of conserved molecules which are commonly found in a variety

of microbial species. Phytohormones involved in the pathogen responses are ethylene and jasmonates. Ethylene biosynthesis seems to be induced with gene expression levels of ACC synthase being up-regulated in both lumichrome treated *Lotus* and tomato plants. Other responses include the induction of tryptophan biosynthesis, the accumulation of secondary metabolites and the induction of plant genes coding for defensins (Brader *et al.*, 2001; Glazebrook, 2005; Wasternack, 2007; Doehlemann *et al.*, 2008). These are all responses observed in this study. Furthermore, an induction of genes associated with the shikimate pathway was observed by Doehlemann *et al.* (2008). Some genes in the shikimate pathway have also been pointed out as activated, although moderately, by nitrogen deficiency (Weaver and Herrmann, 1997; Scheible *et al.* 2004; Lillo *et al.*, 2008). Again, with the up-regulation of DAHP synthase in both *Lotus* and tomato lumichrome treated plants, this paralleled their observation. Additionally, fungus induces the formation of the amino acid GABA, whose role is to protect the plant from oxidative stress (Solomon and Olivier, 2002). In both *Lotus* and tomato, GABA seems to play a significant role as the induction of the genes for GABA permease and glutamate decarboxylase was observed.

However, in the majority of the cases it was accompanied by decreases in plant growth as defence must come at a cost and involves a massive redistribution of energy towards the defence response. Indeed, a common feature in many microarray papers that profile response to a particular pathogen is a gene list containing primary metabolism genes that likely play a role in providing energy for the resistance response (Bolton, 2009). Generally, the central metabolic pathways and other energy generating pathways are up-regulated during defence responses. In contrast to this, lumichrome treated plants seem to down-regulate glycolysis with the result of increased plant growth. The question then arises as to where the necessary energy stems from to maintain and even increase plant growth. The degradation of fatty acids during β-oxidation could be another potential energy source during plant defence responses. The complete oxidation of a fatty acid molecule generates a significant amount of ATP equivalents by producing one NADH, one $FADH_2$ and one acetyl-CoA for each round of the cycle (Lynen, 1955) and the pathway has been shown to be up-regulated during the resistance response to several pathogens (Bolton *et al.*, 2008; Bolton, 2009; Schenk *et al.*, 2003).

Lumichrome has been shown to act as a photosensitizer (Huang *et al.*, 2006), which is a chemical compound that readily undergoes photoexcitation and then transfers its energy to other molecules. Photoexciation is the mechanism of electron excitation, which is the movement of an electron to a higher energy state, through the absorption of a photon.

Therefore, lumichrome is involved in the transfer of energy and consequently shifts in energy metabolism can be expected. Possibly, lumichrome has a specific binding affinity to a specific protein, which plays a role in generating ATP/$FADH_2$/NADH. It is not clear whether lumichrome exerts its effect in the roots or if it is transported to the shoot where it possibly undergoes photoexcitation, transferring energy to perform necessary reactions and even influence the photosynthesis apparatus of the plant. Furthermore, the primarily non-toxic lumichrome was identified to be efficient in transferring excitation energy to substrates and oxygen (Grininger *et al.*, 2006). When exposed to light, photosensitizers such as lumichrome generate singlet oxygen (Huang *et al.*, 2006). Kim *et al.*, (2008) investigated the response of plants to singlet oxygen and found that within 30 min of the release of the singlet oxygen, 50 genes encoding putative transcription factors showed a threefold increase in expression. These included ethylene responsive factors, WRKY transcription factors, zinc finger proteins and several DNA-binding proteins which interestingly, have shown differential expression in this present study of lumichrome. ROS have a large diversity of biological activities including defence reactions against pathogens and the regulation of cell expansion and development which are both themes very much present in this study.

Conclusion

In conclusion, lumichrome elicits growth at nanomolar concentrations in *Lotus* and tomato. The treatment with lumichrome caused complex changes in gene expression, predominantly effecting genes associated with RNA regulation of transcription, signaling, protein synthesis/ degradation/modification and stress and defence. Overall, one of the most significant observations was that for the first time to our knowledge, six genes related to defence and pathogen responses were identified that are concurrently expressed in both *Lotus* and tomato. Through identifying a small number of genes involved in mediating the growth stimulation, these can be used for their functional analysis in the future using reverse genetics to provide more insight into the molecular mechanisms that are triggered by lumichrome as a plant growth promoter.

Future work

This study has opened the door for further investigation into the mechanism involved in lumichrome mediated growth promotion. The most obvious priority for future work is to investigate the six genes that were concurrently expressed in both *Lotus* and tomato through more targeted analysis. To assess the precise involvement of phytohormones, the levels of the main phytohormones should be determined in lumichrome treated and untreated plants. Specifically the results regarding ethylene and ABA should be of great interest. As oxidative stress appears to play a possible role, ROS and its detoxifying enzymes can be measured to determine the exact extent of its role in the plant response to lumichrome. Photosynthesis measurements would greatly contribute to the understanding of how the photosynthesis apparatus is affected by lumichrome. As the growth was most prominent in the roots, root respiration too should be measured. A time course experiment of gene expression, protein and metabolites would ultimately reveal if the response of the plant to lumichrome is immediate on the application of lumichrome and which genes and proteins are induced immediately. Lumichrome seems to influence glycolysis, as the migration in 2D gels was affected for three glycolytic enzymes in tomato and one in *Lotus*. Therefore, targeting the main enzymes of glycolysis in further experiments would make sense. Moreover, nitrogen metabolism seems to be influenced by lumichrome and the various enzymes involved in nitrogen assimilation could be measured as well as total N content. Seeing that the majority of the proteins were down-regulated and the metabolite analysis of *Lotus* showed a general decrease in amino acids, total protein content must be investigated. Additionally, the root exudates can be measured to assess if the speculations around the decreased organic acid - and amino acid content can be verified. In this study, lumichrome has been shown to influence the expression of GAPDH in both *Lotus* and tomato. Therefore, the specific role of post-translational modification needs to be determined. Furthermore, one can determine if lumichrome treated plants are more resistant to pathogens, when compared to untreated control plants. Finding the specific protein that binds lumichrome, for example the homologue in *Lotus* and tomato to dodecins in *Halobacterium salinarum*, would be of great interest in following and discovering the exact pathway that is triggered upon lumichrome application. Investigating whether cell size or cell number is increased in lumichrome treated roots, will possibly clarify the question regarding increases in biomass and growth.

The possibilities are endless, as this is only the beginning of unraveling the mysteries behind the mechanism of lumichrome as a plant growth promoter.

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