DOCTORADO EN CIENCIAS Y BIOTECNOLOGÍA DE PLANTAS

Proteomic study of hairy roots of *Catharanthus roseus* (L.) G. Don treated with methyl jasmonate

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ABREVIATURAS

AOC	Allen oxide cyclase
AOS	Allen oxide synthase
JA	Jasmonic acid
JA-OH	Hydroxyjasmonate
JA-Ile	Jamonic acid isoleucine conjugate
JAR1	JA-ile conjugate synthase
JAs	Jasmonates
LA	Linolenic acid
LOX	Lipoxygenase
MeJA	Methyl jasmonate
OPDA	12-Oxophytodienoic acid
PLA1	Phospholipase A1
PLD	Phospholipase A2
PR	Pathogenesis related protein
2D-PAGE	Two dimensional polyacrylamide gel electrophoresis

CONTENIDO

RESUMEN

El ácido jasmónico y sus moléculas relacionadas comúnmente llamados jasmonatos (JAS), están ampliamente distribuidas en el reino vegetal. Los JAs son reconocidos en la actualidad como moléculas señal, de central importancia en diversos procesos vegetales tales como el desarrollo vegetal y la respuesta a cambios ambientales extremos. Entre estas moléculas, el jasmonato de metilo (MeJA) es utilizado como un potente inductor del metabolismo secundario en diversos modelos vegetales. La participación de los JAs en estos procesos vegetales son bien conocidas en las partes aéreas de la plantas. Sin embargo, nuestro conocimiento acerca de estas respuestas en las raíces es muy limitado. Debido a su estabilidad metabólica y rápido crecimiento, las raíces transformadas con *Agrobacterium rhizogenes* son una alternativa viable para el estudio de este regulador del crecimiento. La acumulación de alcaloides en las raíces transformadas de *C. roseus* al ser tratadas con MeJA sugiere un cambio en la expresión proteica de dicho órgano. En este trabajo se llevó a cabo el análisis proteómico de cultivos de raíces transformadas de *C. roseus* tratadas con MeJA. Se identificaron 58 proteínas, pertenecientes a 11 categorías funcionales. La sobre acumulación de alcaloides fue acompañada por la represión de proteínas pertenecientes al metabolismo de carbohidratos (21%), crecimiento y organización celular (10%), energía (7%) y ciclo celular (3%), mientras que las proteínas sobre expresadas pertenecen al metabolismo de los aminoácidos (12%), modificación proteica y chaperonas (12%) y metabolismo secundario (7%). En este trabajo se hace una propuesta de un modelo que explica dicho comportamiento.

ABSTRACT

Jasmonic acid and related molecules commonly named Jasmonates (JAs) are broadly distributed in the plant kingdom. JAs are currently recognized as major signal molecules in several plant processes which mediate stress response (including pathogen attack) and several developmental processes. Among these signal molecules Methyl jasmonate (MeJA) has been used as a potent elicitor of secondary metabolism in a broad range of plant systems. This involvement of JAs in plant process is well documented in the aerial parts of the plant. However the knowledge about this response in the below ground planted organs is poorly understood. Because of the rapid growth, genetic and biochemical stability, the hairy roots *in vitro* cultures established with *Agrobacterium rhizogenes* is one alternative for the study of this growth regulator. The over accumulation of alkaloids in the hairy roots treated with exogenous MeJA suggests a change in the protein expression in this organ. In the present work proteomics survey of hairy roots treated with MeJA was made. 58 differentialy expressed proteins were identified, belonging to 11 functional categories. The over accumulation of alkaloids was correlated with the repression of proteins belonging to carbohydrate metabolism (21%), cell growth and organization (10%), energy (7%) and cell cycle (3%), whereas amino acid metabolism (12%), protein modification and chaperons (12%) and secondary metabolites (7%), were highly expressed. A model for this behavior is proposed in the present work.

INTRODUCTION

Jasmonic acid and its cyclopentane derivatives as well as cyclopentenones are synthesized from the octadecanoid lipid pathway and widely distributed throughout the plant kingdom (Gfeller *et al.*, 2006). They are structurally related to prostaglandins and are recognized as signal molecules with important roles in a variety of biological processes such as plant development and environmental stresses (Turner *et al.*, 2002; Howe, 2004). This signal molecules referred as Jasmonates are potents regulators of genes involved in several plant processes. Over the last decade, the JA signaling pathway has been studied extensively in dicot plant species, such as Arabidopsis (*Arabidopsis thaliana*), tomato (*Solanum lycopersicum*), and tobacco (*Nicotiana tabacum*), and to a somewhat limited extent in monocot plants, such as barley (*Hordeum vulgare*) and rice (*Oryza sativa*). Although much remains to be learnt, both forward and reverse genetic studies, particularly in Arabidopsis, have greatly expanded our understanding of the potential roles of JAs in plants.

Mutants in JA biosynthesis as well as in signal transduction downstream of biosynthesis have been identified (Wasternack, 2007; Delker *et al.*, 2006). Theoretically, mutants with altered JA levels could be affected in biosynthesis, degradation or modification of JAs, or in the regulation of these metabolic steps. So far, several mutants blocked in one of the biosynthetic enzymes, as well as a transgenic line with altered JA modification, have been isolated (Delker *et al.*, 2006). Downstream of JA biosynthesis, mutants that show either impaired or constitutive JA responses could be expected. Recessive traits of JA insensitivity can be caused by a mutation

in a positive regulator of JA signaling and vice versa; constitutive JA-response phenotypes result from the mutation of a negative regulator. Mutants of both types have been found. The analysis of these mutants has also contributed to unravel the role of JAs as signal compound in several plant processes. The use of protein and gen microarrays have allowed to identified more genes that are regulated by JAs or the processes that JAs are connected with (Cho *et al.*, 2007). A surprisingly high number of genes have been found by this approach to be co-regulated by JAs and SA (Schenk *et al.*, 2000). Analysis of the expression of these microarray-identified genes in the mutants will further define the signaling pathways regulating the expression of these genes.

Several aspects such as wounding, herbivore, pathogen interactions and developmental processes where JAs are involved are well documented in the aerial parts of the plants (Seo *et al.*, 2001; Gatehouse, 2002). However, our knowledge about plant root defense responses and other interactions is poorly understood. The reason for this lack of information is probably because of the underground roots' localisation, the complexity of the rhizosphere and the few experimental systems for the study on roots. One alternative for root study is the establishment of hairy root cultures with *Agrobacterium rhizogenes* (Flores *et al.*, 1987; Flores & Filner, 1985; Rhodes *et al.*, 1990). The hairy roots have stable expression of their metabolic pathways and have been used for the study of secondary metabolism of several medical plants such as the biosynthetic pathway of terpene indole alkaloids (TIAs) of *C. roseus* (Jung & Tepfer, 1987; Kamada *et al.*, 1986; Rhodes *et al.*, 1986). *C. roseus* (Madagascar periwinkle) is the source of the antitumor drugs vinblastine and vincristine. These TIAs are minor products in the plants, making their isolation elaborate and costly. In addition, the complexity of the biosynthetic pathway of these alkaloids made it an intellectual challenge to resolve. During the study of the metabolic pathway in this plant, methyl jasmonate (MeJA) has been used as elicitor of TIAs

(Vázquez-Flota *et al.*, 1994). For this reason we hypothesized that the change in the proteome of hairy roots by MeJA exposure correlated with the over accumulation and secretion of TIAs. In order to test this hypothesis in the present thesis, a proteomics approach was used. Proteins of *C. roseus* hairy roots challenged with different amounts of MeJA were submitted to 2D-PAGE (two dimensional polyacrylamide gel electrophoresis) and a survey of differentially expressed proteins was made. Q-TOF mass spectrometry analysis of these proteins identified 58 of them, belonging to 11 functional categories. Proteins involved in carbohydrate metabolism (21%), cell growth and organization (10%), energy (7%) and cell cycle (3%) were highly repressed, whereas amino acid metabolism (12%), protein modification and chaperons (12%) and secondary metabolites (7%), were highly expressed. In the same scenario the over accumulation and secretion of TIAs were also observed.

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Chapter 1

JASMONIC ACID AND PLANT ROOT SYSTEM

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1.1. ABSTRACT

Jasmonic acid and related compounds collectively referred as jasmonates, form a family of cyclopentanone derivatives synthesized from linolenic acid via the octadecanoid pathway. Jasmonates as signal molecules regulate several aspects of plant development, biotic and abiotic stress responses. The occurrence of Jasmonates and the octadecaonoid pathway in the whole plant is suggested. However in the last decades, studies have focused on the aerial parts, discriminating the aspects where jasmonates are involved in the belowground organs. The present review is focus on the occurrence of the octadecanoid pathway in plant roots and the implication of jasmonates in root growth inhibition, tuber formation, secondary metabolism, root defense mechanisms and exudation of plant roots.

1.2. INTRODUCTION

Phytohormones are instrumental in regulating metabolic, development and defensive processes in plants. At the time of its first description (Demole *et al.*, 1962), the methyl ester of jasmonic acid (MeJA) was considered as one of many plant secondary metabolites with a possible application in the perfume industry. Plant extracts were quickly replaced by synthetic MeJA and its cheaper analogs, such as methyl dihydrojasmonate, which is now a very popular commercial fragrance component under the name of hedione. In parallel, studies on the physiological role of jasmonic acid (JA) an related compounds collectively referred as jasmonates (JAs)[1] originated from octadecanoid pathway, revealed the complex nature of the plant defense systems and promoted JAs to the rank of true plant hormones, which mediate in various aspects of development and stress responses (Turner *et al.*, 2002; Creelman & Mullet, 1997a; Aerts *et al.*, 1994; Gantet *et al.*, 1998; Rijhwani & Shanks, 1998; Lee-Parsons *et al.*, 2004). In the last two decades the number of publications have increased (Figure 1.1) leading to new insights about the regulation mechanism of JAs perception. It has been shown that JAs function as physiological regulator in a wide range of biological processes, including development (tendril coiling, tuberization, germination, root growth, fertility, fruit ripening, and senescence) and defense (against pathogens and pests, wounding, abiotic stresses, and secondary metabolism)(Vick & Zimmerman, 1984; Feussner & Wasternack, 2002; Wasternack, 2007a; Creelman & Mullet, 1997a). The mode of action of JAs has been investigated traditionally by analysis of the effects of exogenous application of these compounds (Creelman & Mullet, 1995), including the identification of JAs responsive genes (Creelman & Mullet, 1997b) and determination of their

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[1] In this paper we used the term jasmonates to refer to the all bioactive intermediates of jasmonic acid biosynthetic pathway, as like derivatives.

expression and responsive promoter elements (Menke *et al.*, 1999; Van der Fits & Memelink, 2000; Van der Fits & Memelink, 2001). Moreover, JAs biosynthesis has been studied by identification of biosynthetic enzymes, use of inhibitors and determination of endogenous JAs levels (Turner *et al.*, 2002). Several mutants defective in JA biosynthesis and signaling have been isolated and their phenotypes shed new light on the role of JAs. Recently with the new technology of transcriptomics and proteomics is possible to undertake a global approach helping in a better understanding of the role of JA and its regulatory networks in plants.

In spite of the valuable insight into root biochemistry, development and other functions that has led the recent interest in root biology research (Flores *et al.*, 1999; D'Auria & Gershenzon, 2005), the study of the biological effect of JAs in belowground organs have discriminated against aboveground organs. However, the potential of JAs as an above and belowground regulator is indicated by the fact that, when JA is applied in leaves of *Nicotiana silvestris* it seems to be transported to the roots where it induces nicotine synthesis (Zhang & Baldwin, 1997). Furthermore, application of JA (or MeJA) to roots induces genes related with defenses in shoot (Baldwin, 1996; Van Dam *et al.*, 2004; Van der Putten *et al.*, 2001), providing additional evidence for its key role in root and shoot interactions. The detection of some enzymes of octadecanoid pathway and JAs in plant root suggests the possibility of JA production in this organ (Hause *et al.*, 2002; Bell *et al.*, 1995; Hause *et al.*, 2000; Pedranzani *et al.*, 2003). Like in aboveground organs, JAs have the ability to change the profiled gene expression of plant roots (Choi *et al.*, 2005). Several root genes are up and down regulated by JAs (Reinbothe *et al.*, 1994b; Herrmann *et al.*, 1989; Parthier, 1991; Cho *et al.*, 2007; Reinbothe *et al.*, 1994a; Rakwal & Komatsu, 2000). However, poor attention is focused to the proteome level; hence, the changes that occur at the protein level are not understood. It is currently unclear whether phosphoproteome or

glycoproteome patterns of roots are modified by the JAs. Moreover no knowledge has been generated about involvement of JAs in root protein exudation mechanism. A related concern is that substantial regulation of cellular events can occur at the protein level with no apparent changes in mRNA abundance (Jacobs *et al.*, 2000). The posttranslational modification of proteins can result in a dramatic increase in protein complexity without a concomitant change in gene expression(Jacobs *et al.*, 2000). Proteomics may be the most promising technique for identification of proteins that are involved in roots biological processes mediated by JAs. Quantitative and qualitative analysis of the plant root proteome is an important step towards further characterization of differentially expressed proteins and the elucidation of the mechanisms underlying the development and biological functions of roots. The following review will cover some evidence about the occurrence of the octadecanoid pathway in plant roots as well as the biological processes of roots mediated by JAs.

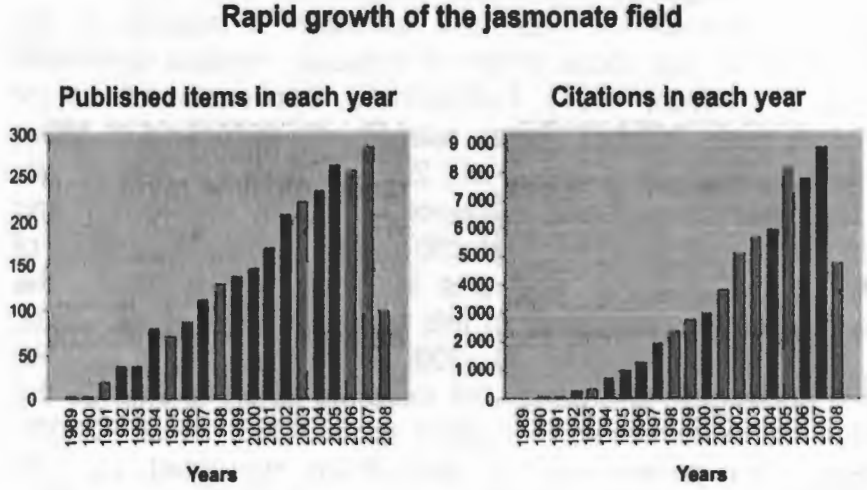

Figure 1.1. *Publish items and citation of jasmonates papers in each year. Topic = jasmonate; Timespan = 1989 – June, 2008. The information was obtained in the ISI Web of Knowledge citation report, http://www.isiwebofknowledge.com/.*

1.3. BIOSYNTHETIC PATHWAY IN ROOTS

The pathway of the octadecanoid biosynthesis was elucidated in the early 80s by Vick and Zimmerman (1984). The first three steps are located in the chloroplast (FDA1, LOX, AOS and AOC), peroxisome (OPR, ACX, MFP and KAT) and the modifications of terminal product are made by citoplasmic enzymes (Figure 1.2). So far, it has been reported that leaves are the sites in which JA biosynthesis occurs (Creelman & Mullet, 1997a); although there is evidence of a possible biosynthetic pathway in roots (Figure 1.5)(Pedranzani *et al.*, 2003). One piece of evidence is the high expression of LOX and AOC detected in roots (Bell *et al.*, 1995; Hause *et al.*, 2000; Hause *et al.*, 2002). Hence, there is a possibility of occurrence of the octadecanoid pathway in roots, excluding the possibility of a JA transport from the shoot and also avoiding the production of other signals like systemin. But we cannot rule out the possibility of the basipetal translocation of some intermediates of the octadecanoid pathway.

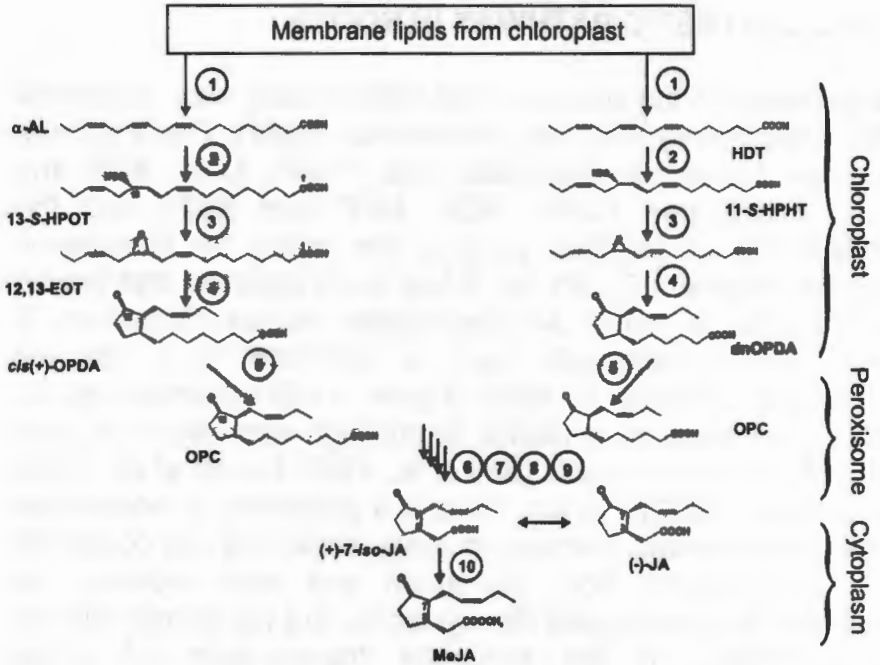

Figure 1.2. *Metabolic pathway of jasmonic acid. The involved enzymes are indicated with numbers and intermediates with the corresponding abbreviation. 1, phospholipase A1; 2, lypoxygenase (LOX); 3, allene oxide synthase (AOS); 4, allene oxide cyclase (AOC); 5, OPDA reductase; 6, acyl-CoA synthase; 7, acyl-CoA oxidase; 8, multifunctional protein (MFP); 9, acetyl-CoA C-acyltransferase (KAT) and 10, metlhyl transferase. Modified figure from Turner et al (2002).*

1.3.1. RELEASE OF LINOLENIC ACID

The biosynthesis of JAs begins with the release of linolenic acid (18:3) (9Z, 12Z, 15Z-octadecatrienoic acid; LA) or rouganic acid (16:3) (7Z, 10Z, 13Z-hexadecatrienoic acid) from plasmatic membrane of chloroplast. This release is catalyzed by the enzyme phopholipase A_1[2] (NC), (NS: phosphatidylcholine 1-

[2] The first time that names an enzyme gives the systematic name (SN) and the common name (CN), later, as well as in the figures, is given only the common name. For the names of enzymes and their nomenclature the rules have been followed of nomenclature Committee of the International Union of

acylhydrolase; EC 3.1.1.32, reaction 1, figure 1.2) (Narvaez-Vasquez *et al.*, 1999; Farmer & Ryan, 1992; Mueller *et al.*, 1993). Correlation between the increase of phospholipase A_1 activity by elicitation (Roy *et al.*, 1995) and rapid release of LA preceding the accumulation of JA (Mueller *et al.*, 1993) and the characterization of the *A. thaliana* mutant, dad1, male sterile (Ishiguro *et al.*, 2001) supported the evidence of the existence of phospholipase A_1 (PLA1) which releases LA for octadecanoid biosynthesis from membrane lipids. *dad1* was isolated from seedling male sterile mutants, which could be rescued by LA or JA application. The mutation defined an open reading frame, which encodes a lipase that hydrolyses phospholipids in a sn-1-specific manner, indicating that *DAD1* is a chloroplastic PLA_1. PLA_1 gene expression is restricted in filaments of stamens immediately before flower opening (Ishiguro *et al.*, 2001). However, phospoholipase A2 (PLD) is constitutively present in total protein extracts from leaves and roots (Frank *et al.*, 2000). The expression was localized mostly in root vascular tissues (Lee *et al.*, 2003). Interesting, PLD also has been suggested to be involved in plant defense responses to wound stress and pathogen elicitors (Narvaez-Vasquez *et al.*, 1999; Farmer & Ryan, 1992; Mueller *et al.*, 1993). Therefore the release of LA is also possible by PLD (Narvaez-Vasquez *et al.*, 1999; Farmer & Ryan, 1992; Mueller *et al.*, 1993). Since JA induces the expression of different sets of genes in roots and leaves, it suggests the presence of a complex, organ-specific transduction network that regulates the effects of JAs on plant gene expression (Dammann *et al.*, 1997). The first possible branch comprises the requirement of PLA_1 (Turner *et al.*, 2002) and the second, is the requirement of the PLD (Narvaez-Vasquez *et al.*, 1999; Farmer & Ryan, 1992; Mueller *et al.*, 1993).

Biochemistry and Molecular Biology (NC-IUBMB) (http://www.chem.qmul.ac.uk/iubmb/enzyme/).

1.3.2. LIPOXYGENASE (LOX)

Lipoxigenases are widely distributed in animals, in man and in higher plants (Siedow, 1991). Proteome analysis of chloroplast stromal proteins showed that monomeric LOX2 protein is 1.5% of the total amount of protein, thus making it among the group of abundant enzymes of the Calvin cycle (Peltier *et al.*, 2006). Among the six LOX genes of *A. thaliana* (Feussner & Wasternack, 2002), the 13-LOX2 (NS: linoleate:oxygen 13-oxidoreductase; EC 1.13.11.12; reaction 2, figure 1.2) encoded by *LOX2* seems to be involved in JA biosynthesis. The 13-LOX2 catalyze oxygen insertion at carbon 13 of the LA carbon backbone, leading to (13S)-hydroperoxyoctadecatrienoic acid (13-HPOT). Antisense suppression of an Arabidopsis stroma-localized plastid *13 - LOX2* also suppressed wound-induced JA formation but did not affect male fertility. Apparently, *LOX2* is required for wound-induced JA formation, but is not required for JA-dependent pollen and stamen development (Bell *et al.*, 1995). In potato roots two other *LOX* genes named *LOX1* and LOX3 were identified, both *mRNAs* are increased upon wounding and JA treatment. However, only the LOX3 produces the JA precursor, 13-hydroperoxylinolenic acid, as the major product of the action of LOX3 on linolenic acid (Royo *et al.*, 1996). This suggests the involvement of LOX3 in JA biosynthesis in root tissues.

1.3.3. ALLEN OXIDE SYNTHASE (AOS)

Allene oxide synthase (NS: (9*Z*,11*E*,14*Z*)-(13*S*)-hydroperoxyoctadeca-9,11,14-trienoate 12,13-hydro-lyase; AOS; EC 4.2.1.92; reaction 3, figure 1.2) catalyses the dehydration of 13-hydroperoxy-octadecatrienoic acid to an unstable epoxide 12, 13(S)-epoxy-9(Z), 11, 15(Z)-octadecatrienoic (12,13-EOT), which is thought to be converted to OPDA by allene oxide cyclase (AOC). The Arabidopsis AOS promoter is activated by a variety of signals including jasmonic acid, wounding, OPDA and SA, indicating that regulation of the

expression of the AOS protein might exert a major control on JA signalling (Laudert & Weiler, 1998). Moreover, the highly unstable AOS product and its single gene for AOS in the *A. thaliana* genome (Kubigsteltig *et al.*, 1999), is regarded as a step in the regulation of JA biosynthesis (Laudert & Weiler, 1998; Park *et al.*, 2002b). Plastid localization of AOS has been shown by immunolocalization and import studies, even for the barley AOSs that lack the transit peptides (Maucher *et al.*, 2000). However, cloned potato 9-AOS is associated with amyloplasts and leucoplasts in below ground organs of potato (Stumpe *et al.*, 2006). Previously, low amounts of AOS *mRNA* were detected in potato roots (Sivasankar *et al.*, 2000). Similar results were observed in root tips of young root barley (Maucher *et al.*, 2000). In addition, constitutive levels of the AOS protein have also been observed in *A. thaliana* roots (Laudert & Weiler, 1998). The involvement of a single AOS protein in the biosynthetic pathway of JA in root plastids might be expected.

1.3.4. ALLENE OXIDE CYCLASE (AOC)

The enzymatic conversion of unstable 13, 13 EOT to (9S,13S)-12 oxo-(10,15Z)-phytodienoic acid (p. ej. cis(+)-OPDA) is achieved by the allene oxide cyclase (Kitajima *et al.*, 1991)-(13*S*)-12,13-epoxyoctadeca-9,11,15-trienoate isomerase (cyclizing); AOC; EC 5.3.99.6; reaction 4, figure 1.2). So far, more than 58 *AOC* sequences have been deposited in the database and partially characterized by functional assays (Delker *et al.*, 2006). The phylogenetic tree analysis for AOCs revealed small, but clearly distinct gene families for monocotyledonous and dicotyledonous plants (Stenzel *et al.*, 2003). AOC is considered an important regulation point because of the establishment of the ultimate enantiomeric structure of the natural occurring of JA. Four *AOCs* from *A. thaliana* name AOC1, AOC2, AOC3 and AOC4 are localized in the chloroplast (Stenzel *et al.*, 2003). The AOCs are expressed locally and systemically in response to wounding, with preferential expression of *AOC2* (Stenzel *et al.*, 2003). Recent

analysis of transgenic lines carrying the GUS reporter gene under the control of the individual AOC promoters revealed no-redundant promoter activities during distinct stages of development (Delker *et al.*, 2006). The AOC4 protein shows activity in the root tip throughout root development, whereas AOC3 first shows activity in the meristematic and elongation zone seven days after germination, and the activity shifts into the stele of the elongation zone about 14 days after germination (Delker *et al.*, 2006). These results are similar with a uy previous work where AOC mRNA was remarkably accumulated in tomato hairy roots. AOC protein was specifically detected in the parenchymatic cells of all root vascular bundles (Abdala *et al.*, 2003). The product of AOC, OPDA and its methylated derivative, OPDAMe, were detected in hairy roots. Moreover under salt stress both compounds showed higher levels of JA and MeJA (Abdala *et al.*, 2003). These findings indicate that at least one step in the JAs synthesis may take place in the root organ (Hause *et al.*, 2000).

1.3.5. OPDA REDUCTASE

OPDA reductase (NS: 8-[(1*R*,2*R*)-3-oxo-2-{(*Z*)-pent-2-enyl}]octanoate:$NADP^+$ 4-oxidoreductase; OPR3; EC 1.3.1.42; reaction 5, figure 1.2) catalyses the reduction of OPDA to 3-oxo-2-(2 - (Z)-pentenyl)- cyclopentane-1 octanoic acid (OPC-8:0). Although Arabidopsis contains at least two other *OPR* genes, named *OPR1* and *OPR2,* which their transcription are induced by wound (Biesgen & Weiler, 1999), their protein products do not catalyze the reduction of OPDA (Schaller *et al.*, 2000). The perosixomal location of OPR3 protein in leaves was shown by immunocytochemical detection, as well as, expression analysis of OPR3 fused to GUS reporter gene confirmed this subcelular location (Stintzi & Browse, 2000). For the import of OPDA or its CoA ester into peroxisomes, there is some evidence for transport by the ABC transporter COMATOSE (CTS) (Theodoulou *et al.*, 2005). Parallel pathway of OPDA import by ion trapping has also been suggested

(Theodoulou *et al.*, 2005). Recently it was reported the cloning and characterization of a cDNA (*SiOPR1*) encoding a putative 12-oxophytodienoic acid reductase 1 from foxtail millet by RACE methods. Sequence analysis showed that *SiOPR1* encoded a polypeptide of 374 amino acids with a predicted molecular mass of 41.9 kDa and pI of 5.14. Multiple sequence alignment showed that OPR1 protein was highly conserved among gramineous crops. RNA gel blot analysis results indicated that *SiOPR1* was up-regulated by osmotic stress, and its expression was limited in the roots of foxtail millet (*Setaria italic*) (Abdala *et al.*, 2003).

1.3.6. *β*-OXIDATION

The last step of JA biosynthesis includes three rounds of β-oxidation, in which the carboxylic acid side chain is shortened. This idea rose with feeding experiments in tomato with compounds carrying different lengths of carboxylic acid side chains, wich revealed that only even numbered OPC derivatives were converted to JA (Miersch et al., 2000). Recently, genetic analysis have revealed recently that fatty acid β-oxidation enzymes are active in JA biosynthesis (Castillo *et al.*, 2004; Delker *et al.*, 2006). β-oxidation pathway comprised the acyl-CoA synthase (SN: acyl-CoA:malonyl-CoA *C*-acyltransferase (decarboxylating, oxoacyl- and enoyl-reducing); EC 2.3.1.86; reaction 6, figure 1. 2), acyl-CoA oxidase (SN: acyl-CoA:oxygen 2-oxidoreductase; EC 1.3.3.6; ACX; reaction 7 figure 1.1) and a multifunctional protein (MFP; reaction 8 de la figure 1.2) that includes an activity of enoyl-CoA hydratase (SN: (3*S*)-3-hydroxyacyl-CoA hydro-lyase EC 4.2.1.17) and β-hydroxyacyl-CoA dehydrogenase (SN: (*S*)-3-hydroxyacyl-CoA:NAD^+ oxidoreductase; EC 1.1.1.35), and the last step is catalyzed by the ketoacyl-coenzyme A thiolase (SN:acyl-CoA:acetyl-CoA *C*-acyltransferase; KAT; EC 2.3.1.16; reaction 9, figure 1.2). In *A. thaliana*, ACX1 and KAT2 expression is up regulated upon wounding and antisense expression of ACX1 and KAT2 led to JA biosynthesis (Castillo

et al., 2004). Several mutants affected in ACX genes exhibit reduced fatty acid acyl-CoA oxidase activity and enhanced resistance to the endogenous auxin analogue indole-butyric acid (IBA) due to the role of fatty acid acyl-CoA oxidase in IBA formation (Adham *et al.*, 2005). Consequently, mutants affected in the ACX gene may have JA deficiency upon wounding due to the involvement of ACX and β-oxidation step in JA biosynthesis. In the peroxisomes, the β-oxidative steps take place only with the corresponding CoA ester and the synthesis of OPDA–CoA ester is mediated by the 4-coumarate:CoA ligase-like (4-Cl-like) enzymes encoded by a small gene family in *A. thaliana* (Schneider et al., 2005). So that, the metabolic pathways of β-oxidation is suggested to occur specifically in peroxisomes (Cooper & Beevers, 1969). Some clues indicate the induction of β-oxidation in roots. This pathway is up regulated in detached maize root tips (Dieuaide *et al.*, 1992). Recently, three isogenes of *ACX* were identified in the rice genome. The deduced proteins of *OsACX*1, *OsACX*2 and *OsACX*3 consisted of 669, 699 and 685 amino acid residues, respectively. The results from reverse transcriptase-PCR indicated that only *OsACX*1 was expressed in roots (Kim *et al.*, 2007). 3-ketoacyl thiolase (*PED1*) was also expressed in roots (Charlton *et al.*, 2005).

Homeostasis between various metabolites is a common mechanism in plants to sustain the level of active hormones such as auxins, cytokinines and gibberellins. In the case of JA, seven different metabolic routes have been suggested by identification of the corresponding products, but only a few genes have been cloned so far (Figure 1. 3). Some of these reactions might lead to the irreversible inactivation of JA, whereas others might be reversible and used for transport or storage. Each product has their own particularities and affects the range of signalling activities of the molecule. Nevertheless only MeJA, JA-OH and isoleucine conjugate (JA-Ile) were detected in roots so far (Abdala *et al.*, 2003). The seven metabolic routes for JA are described below.

1. Methylation of JA: it is carried out by a specific methyl transferase (*S*-adenosyl-L-methionine:jasmonate *O*-methyltransferase; JMT; EC 2.1.1.141; reaction 10, figure 1.2)(Seo *et al.*, 2001). Because of the volatile properties of MeJA, it could mediate intracellular and intercellular signalling, and could also function as an airborne signal mediating intra-and interplant communications in defence (Seo *et al.*, 2001).

2. Decarboxylation of JA: A further round of β-oxidation of JA produces *cis*-jasmone (Koch & Lehmann, 1997). This molecule is a common component of plant volatiles which overproduction provides more resistance to insects (Birkett *et al.*, 2000).

3. There is not detailed study, but the reduction of keto group of the cyclopentanone ring has indicated the occurrence of cucurbic acid and its derivatives in various plant species (Sembdner & Parthier, 1993).

4. Formation of jamsonic acid JA-Ile: it is carried out by a JA-Ile conjugated synthase (JAR1) (Staswick *et al.*, 2002; Staswick & Tiryaki, 2004), upon adenylation at the carboxylic side-chain of JA by the AMP-transferase activity of JAR1. Recently a putative receptor of JA-Ile was cloned (JAZ proteins, see below) which binds JA–Ile during interaction with COI1 (F-box protein affected in the coronatine insensitive mutant *coi1*) and contributes to the proteasome-mediated removal of a negative regulator in JA/JA-Ile-induced gene expression points to the regulatory importance of JA amino acid conjugates (Chini *et al.*, 2007; Thines *et al.*, 2007).

5. Hidroxylation of JA (Gidda *et al.*, 2003). These JA modifications were initially found only in solanaceous and it is called tuberonic acid due to the tuber inducing properties (Yoshihara et al., 1989; Helder et al., 1993). 12-OH-JA does

not induce all JA-response genes, suggesting at least partial inactivation of JA signalling by formation of 12-OH-JA (Gidda *et al.*, 2003). Hydroxylation and subsequent sulfonation or sugar-conjugation reactions may provide a mechanism to inactivate excess pools of JA.

6. Conjugation of JA with sugars: JA can be conjugated with sugars to give jasmonoyl-1β-glucose, jasmonoyl-1β-gentiobiose and hydroxyjasmonoyl-1b-glucose (Staswick & Tiryaki, 2004). These compounds have been identified in tobacco BY2 suspension cultures. In contrast to JA, they did not inhibit the G2 phase of cell cycle (Staswick *et al.*, 2002; Staswick & Tiryaki, 2004).

7. Conjugation of the carboxyl acid side chain of JA with ethylene precursor 1-amino cyclopropane-1-carboxylic acid (ACC) (Staswick & Tiryaki, 2004). Trying to define JAR1 like a JA-amino synthetase, Staswick *et al* (2004) identified other JA-conjugates in *A. thaliana*. The synthesis of JA-CC might provide a mechanism to coregulate the availabity of JA and CC for conversion to the active hormone JA and ethylene, respectively

JA conjugates are permanent constituents of plant tissues (Sembdner & Parthier, 1993). Many of them induce a subset of genes compared with JA or MeJA (Kramell *et al.*, 1994). Among the various conjugates, JA-Ile mainly accumulates in leaves (Kramell *et al.*, 1994), flowers (Hause *et al.*, 2000), mycorrhizal roots (Hause *et al.*, 2000; Hause *et al.*, 2003) and hairy roots of tomato (Abdala *et al.*, 2003). This preference is reflected by the enzymatic properties of the JA amino acid conjugate synthase (JAR1), and the *jar1* mutant can be complemented by JA-Ile (Staswick & Tiryaki, 2004). JAR1 belongs to a large gene family encoding enzymes that adenylate a carboxylic acid-containing substrate followed by exchange with a second substrate. In the case of JAR1, AMP is exchanged with an amino acid (Staswick *et al.*, 2002; Staswick & Tiryaki, 2004). The tobacco homologue

of JAR1 has recently been cloned as JAR4 from *N. attenuata* (Kang *et al.*, 2006). Interestingly, JAR4-silenced plants were shown to be more susceptible to *Manduca sexta* attack, indicating the role of JA-Ile in plant defense. Recently SCF^{COI1}–JAZ1 protein complex was indicated as a site of perception of JA-Ile.

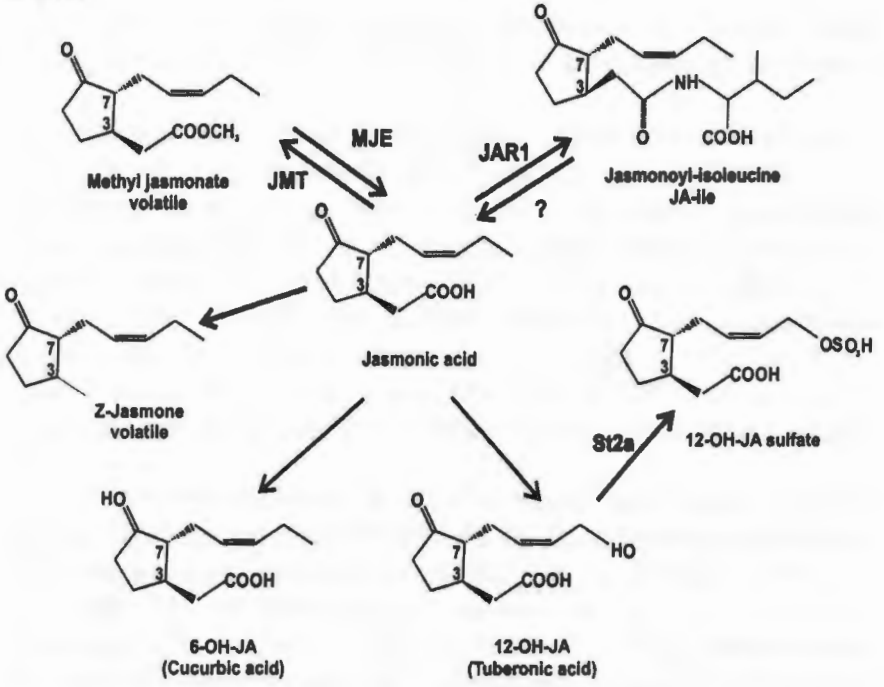

Figure 1.3. *Chemical structure of JA and its derivatives. JA produced from the octadecanoid pathway can be further metabolized by several enzymes. MJE, methyl-JA esterase; JMT, JA carboxy methyltransferase; JAR1, JA Resistance1; ST2a, sulfotransferase. Plant defence responses are typically activated by treatment with MeJA, JA or JA-Ile. Modified figure from Wasternack (2007).*

This discovery provides the basis for understanding the multifaceted effects of jasmonates on plant development and immunity (see below) (Thines *et al.*, 2007). With the exception of JMT and JAR1, the enzymes involved in modifying JA are unknown. This has limited our ability to deduce the function of specific JA derivatives by genetic and transgenic approaches.

By contrast, plant enzymes that form glycosyl esters of IAA have been studied (Szerszen *et al.*, 1993), and a bacterial IAA-Lys synthetase has been characterized (Roberto *et al.*, 1990).

1.4 JASMONATE BIOSYNTHESIS REGULATION

A great body of evidence indicates that three different mechanisms are involved in the regulation of JA biosynthesis:

1. Substrate availability: Transgenic plants over expressing AOS constitutively did not show elevated level of JA, but generated more JA than the wild type upon wounding (Laudert & Weiler, 2000). Moreover, in the fully development *A. thaliana* leaf LOX, AOS and AOC proteins occurs abundantly, but JA and OPDA are formed only upon external stimuli such as wounding (Stenzel *et al.*, 2003; Stintzi *et al.*, 2001). This transient rise of JA takes place before transcription accumulation of JA biosynthetic genes.

2. Positive feedback: treatment of *A. thaliana* leaves to JA activates expression of all JA biosynthetic genes (Stenzel *et al.*, 2003; Castillo *et al.*, 2004). Microarray analysis reveals that five out of 41 genes responding to JA are JA biosynthetic genes, indicating the existence of a positive feedback regulatory system for JA biosynthesis (Sasaki *et al.*, 2001). This confirms the findings of others, that JAs induce transcription of *DAD1*, *LOX2*, *AOS*, *OPR3*, and *JMT* (Laudert & Weiler, 2000; Seo *et al.*, 2001; Ishiguro *et al.*, 2001). Furthermore, mutants having elevated levels of JA exhibit increased AOC protein levels, whereas JA-deficient mutants such as *opr3* contain less AOC protein than wild type (Delker *et al.*, 2006).

3. Tissue specificity: The distinct AOS promoter activities and the no-redundant promoter activities AOC1-ACO4 in *A. thaliana* roots (Delker *et al.*, 2006) strongly suggest tissue specific regulation of JA biosynthesis. Possibly, the

combined activity of a single copy gene AOS with the different activities of the four AOC genes allows a spatially and temporally distinct generation of JA during development in *Arabidopsis*.

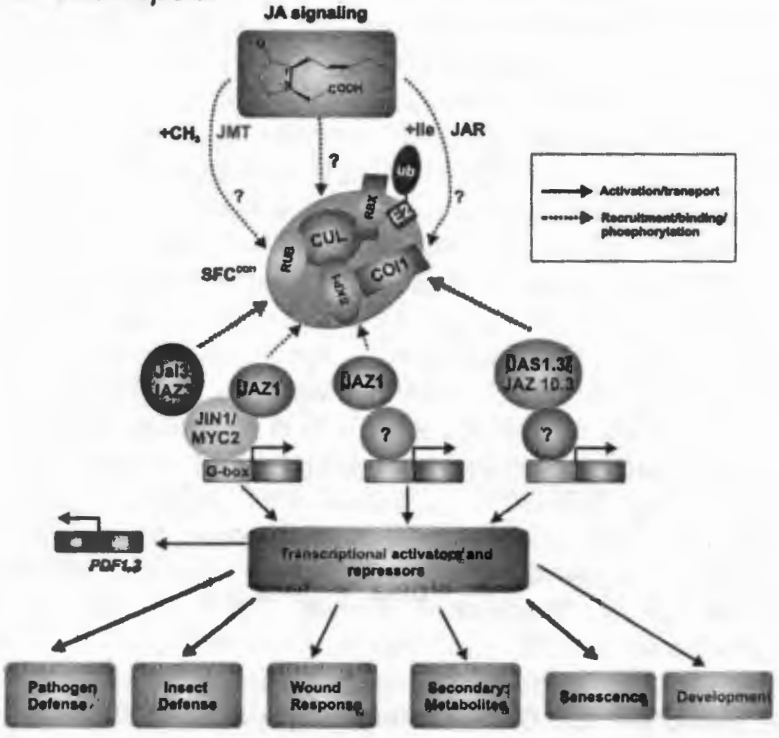

Figure 1.4. *An integrated view of JA biosynthesis and signaling in* A. thaliana. *Biotic and abiotic stresses, such as pathogen and insect attack and wounding, generate signals/elicitors that activate a phosphorylation cascade that regulates JA biosynthesis and signaling. JAZ proteins act as negative regulators of the transcriptional regulator JIN1/MYC2, and their JA- and SCFCOI1-dependent degradation liberates JIN1/MYC2 from repression. JA may also be transported to distal tissue that has not been directly challenged to activate systemic gene expression. Modified figure from Kazan and Manners (2008).*

1.4.1. E3 UBIQUITIN LIGASE REGULATES JA RESPONSES (FIGURE 1.4)

Much about our knowledge of JAs action comes from *A. thaliana* mutants that are deficient in JAs synthesis and/or unable to respond to JAs (Mandaokar *et al.*, 2006; Vijayan *et al.*, 1998; Feys *et al.*, 1994). The *A. thaliana* mutant *coi1* is deficient in all jasmonate responses (Feys *et al.*, 1994). *COI1* gene encodes a 66 kDa protein containing an N-terminal F-box motif, and a leucine rich repeat domain (Xie *et al.*, 1998). F-box proteins are components of SCF complex (Bai *et al.*, 1996), where they function as specific receptors targeting proteins to ubiquitin-mediated proteolysis (Glickman & Ciechanover, 2002). This discovery led to the suggestion that core jasmonate signalling and responses depend on actions of an Skp/Cullin/F-box complex (SCFCOI1), a type of E3 ubiquitin ligase (Deshaies, 1999). This hypothesis has been supported by investigations of additional mutants (Moon *et al.*, 2004; Xu *et al.*, 2002; Lorenzo & Solano, 2005). It is therefore likely that jasmonate signalling involves ubiquitination of specific target proteins by the SCFCOI1 complex and their subsequent degradation by the 26S proteasome (Turner *et al.*, 2002). Unfortunately, extensive genetic screens for positive effectors (Feys *et al.*, 1994; Staswick & Tiryaki, 2004; Jensen *et al.*, 2002; Lorenzo *et al.*, 2004), negative effectors (Xu *et al.*, 2002; Ellis & Turner, 2001) and components downstream of COI1, as well as searches for COI1- interacting proteins (Lorenzo & Solano, 2005; Devoto *et al.*, 2002) have so far failed to identify viable candidates for SCFCOI1 targets. Transcript profiling experiments that have identified jasmonate-responsive (Reymond *et al.*, 2000) and COI1-dependent genes (Devoto *et al.*, 2005) have also failed to provide candidates, or other clues about the precise connection between SCFCOI1 and the jasmonate-regulated transcriptomes. Recently, a family of jasmonate ZIM-domain proteins (JAZ) that includes key components of JAs signalling was discovered (Thines *et al.*, 2007). Molecular and biochemical characterization of *A. thaliana* JAZ1 and tomato orthologues support a model in which JAZ proteins function as repressor of JAs signalling (specifically JA-Ile) and are degraded through the SCFcoi1-

pended 26S proteosome pathway. In other words, JAZ proteins are repressors preventing transcription of JA-Ile responsive genes and the removal of the JAZ proteins by the proteosome pathway allow signaling responses to proceed via increased expression of specific downstream genes (Thines *et al.*, 2007). It is possible that the specificity of interactions between distinct jasmonate compounds, COI1, JAZ proteins, and the downstream transcription or other complexes that JAZ proteins act on determining the outcome of JAs signalling in different tissues. Interestingly, expression analysis in roots of A. *thaliana,* were used to determined the involvement of JAZ protein in JA-Ile perception (Thines *et al.*, 2007). This evidence denote the occurrence of the biosynthetic pathway of octadecanoids and jasmonates in roots as well as key components of JA signaling perception so that we could expect the implication of JAs in several biological processes in roots.

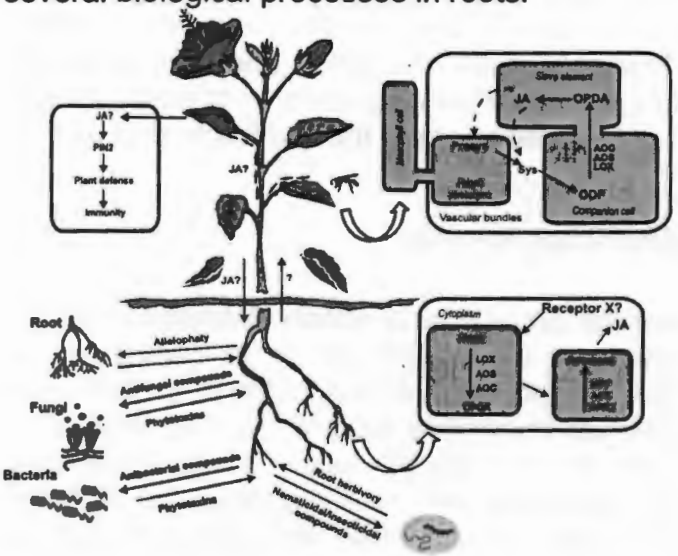

Figure 1.5. *Local and systemic response of JAs in the aerial organs. Specific occurrence of lypoxygenase (LOX), allene oxide synthase (AOS) and allene oxide cyclase (AOC) in the above and below ground organs and root interactions in the rizosphere.*

1.5. JASMONATE INVOLVEMENT IN ROOT PLANT PROCESSES

Interest in root biology research is experiencing a dramatic increase. From a microphytocentric perspective, the availability of *A. thaliana* mutants, along with a sequenced genome, has led to valuable insights in root biochemistry, development, and other functions (Flores *et al.*, 1999; D'Auria & Gershenzon, 2005)
. From an ecological perspective, belowground processes are now recognized as essential components of ecosystem productivity and stability (Van der Putten *et al.*, 2001). The surface area of roots can far exceed that of aerial parts, thus providing tremendous resources for microbes, nematodes, and arthropods in the soil and these organisms are now recognized as drivers of plant diversity and ecosystem functioning (Figure 1.5)(De Deyn & Van der Putten, 2005). However, several aspects such as plant-pathogen interaction and root exudates process in root biology have been neglected. In this section we focus on the present knowledge about the involvement of JAs in plant root biology process.

1.5.1. ROOT GROWTH INHIBITION

This was one of the first physiological effects detected for JA in *A. thaliana* (Staswick *et al.*, 1992). JA or its methyl ester supplemented to the growth medium are active in a picomolar range. Consequently, JA-insensitive mutants such as *coi1*, *jin1*, or *jar1* are similar in root length upon MeJA treatment compared with the untreated wild type. In contrast, mutants such as *cev1*, *cet1*, *joe2* or *cex1* that are characterized by constitutive over-expression of JA-responsive genes due to constitutively elevated JA levels have reduced root length and a stunted growth phenotype similar to JA-treated plants (Ellis & Turner, 2001). Most mutants affected in components of JA- and COI-dependent signal transduction (including transcription factors such as MYC2, the SCF proteasome and its upstream-

acting proteins such as AXR1, JAI4/SGT1b or MPK4) exhibit a reduction in root-growth inhibition (Browse & Somerville, 1991; Wasternack, 2007b). The inhibition of growth by JAs is correlated directly with the blocked of G1, G2 and M phase in cell cycle (Swiatek *et al.*, 2002; Pauwels *et al.*, 2008). These observations might enlighten the long-reported negative impact of JAs on plant growth (Wasternack, 2007a) that is, for instance, also linked to the dwarfed phenotype of *cev1/eli1* mutants (Ellis & Turner, 2001). Recently, in rice using a proteomic approach was possible to correlate several proteins with the root growth inhibition (Cho *et al.*, 2007). Antioxidant systems, cellular respiration, and defense-related proteins, protein modification, chaperon, and cell wall and biogenesis comprised 74% of the total identified proteins whose expressions were altered by JA. Several enzymes from primary metabolism and ATP synthesis were decreased in rice seedling roots. Suggesting that the inhibition of growth in JA treated rice might have resulted from the reduction energy productivity. Proteins like β-5 tubulin and β-tubulin R2242 were also increased and decreased, respectively (Cho *et al.*, 2007). Interestingly, β-Tubulin is the basic component of microtubulines, which play important roles in cell division and elongation in plants. The inhibition of polymerization of β-tubulin by the toxin of rice seedling blight reduces root growth and finally causes plant death (Koga *et al.*, 1995). Moreover, in inhibited root growth of rice was identified a putative actin depolymerizing factor which is 100% identical to the actin factor-depolymerizing factor 4 (ADF-4)(Cho *et al.*, 2007). ADF-4, one of the smallest actin-binding proteins that regulates actin dynamics in cell, enhances the turnover rate of actin and interacts with actin monomers as well as actin filaments (Feng *et al.*, 2006). Putative actin which is 100% identical to actin-2 was also increased. This protein is an important determinant in cytoplasmic streaming, cell shape determination, cell division, organelle movement, and extension growth (Feng *et al.*, 2006). These results may provide a crucial clue for understanding why root growth is inhibited by JAs.

1.5.2. TUBER FORMATION

12-Hydroxyjasmonate (12-OH-JA), also known as tuberonic acid, was first isolated from *Solanum tuberosum* and was shown to have tuber-inducing mainly in *Solanaceaen* family. However, JA, MeJA, 11-OH-JA, OPDA and JA-Ile were also identified during tuber formation of *Solanum tuberosum*, but only the 12-OH-JA and 11-OH-JA showed interesting variations in the endogenous content during the transition of stolons to tubers (Feng *et al.*, 2006). JA and 12- OH-JA decreased in the apical region but remained high in stolons during tuberization. Thus, the apical region might be a site of JAs-utilization or metabolization and stolons might supply JAs to that region. The content of 12-OH-JA was higher than that of 11-OH-JA in all stages, both in apical regions and stolons. However, these compounds showed a different time-course in the apical region, while 11-OH-JA increased, 12-OH-JA decreased (Feng *et al.*, 2006). On the other hand a sulfate conjugated of 12-OH-JA was found to accumulate in the Jamaican feverplant (*Tribulus cistoides*), a member of the Zygophylaceae family (Gidda *et al.*, 2003). This is the only report on the occurrence of a conjugate of 12-OHJA in a plant species that does not produce tubers. Furthermore, it was the first report of the occurrence of a jasmonate conjugated with a sulfonate group. Interesting 12-OH-JA has been detected in *A. thaliana* and identified as the substrate of a sulfotransferase (AtST2a), one of the 18 sulfotransferases occurring in the Arabidopsis genome (Gidda *et al.*, 2003). In mammals, it is well recognized that the sulfonation reaction plays an important role in the modulation of the biological activity of a number of compounds, such as steroids and thyroid hormones, and catecholamine neurotransmitters (Hobkirk, 1993). Sulfonate conjugation not only facilitates transport and excretion of hydrophobic molecules by increasing their water solubility, but it abolishes the biological activity of hormones such as estrogens. The characterization of brassinosteroid sulfotransferase (ST) from *Brassica napus* demonstrated that plants like animals use the

sulfonation reaction to control the biological activity of hormones (Rouleau *et al.*, 1999). For instance root growth inhibition by JA/ MeJA was the first phenotype observed for this type of compound (Dathe *et al.*, 1981) and was frequently used together with germination inhibition in testing JA/MeJA responses, but 12-OH-JA and 12-HSO4-JA are inactive with respect to the inhibition of germination and root growth (Hobkirk, 1993). This suggested that JA/MeJA can become inactive by its hydroxylation. In other words the metabolic conversion of JA by hydroxylation and sulfation leads to a fine-tuning of JA signaling, underlaying a switch-off in JA signaling.

1.5.3. SECONDARY METABOLISM

Roots have a great potential to produce a great variety of secondary metabolites (Bais *et al.*, 2001). In Solanaceus family, secondary metabolites are synthesized in roots and are transported into the aboveground parts of plants. Hence, the pattern of alkaloids found in the leaves is determined by production in the roots (Waller & Kowacki, 1978; Hartmann *et al.*, 1989). One alternative to study the root secondary metabolism is the establishment of hairy root cultures with *Agrobacterium rhizogenes* (Flores & Filner, 1985; Flores *et al.*, 1987; Rhodes *et al.*, 1990). The hairy roots have stable expression of their metabolic pathways and have been used for the study of secondary metabolism like regulation and biosynthesis of alkaloids, flavonoids and sesquiterpenes in medicinal plants (Kamada *et al.*, 1986; Kamada *et al.*, 1986; Rhodes *et al.*, 1986; Loyola-Vargas & Hernández-Sotomayor, 2003; Loyola-Vargas, 1993; Moreno-Valenzuela *et al.*, 1998; Moreno-Valenzuela *et al.*, 1999; Moreno-Valenzuela *et al.*, 1999; Peraza-Sánchez *et al.*, 1998; Vázquez-Flota *et al.*, 1994; Canto-Canché & Loyola-Vargas, 1999). *C. roseus* (Madagascar periwinkle) is the source of the antitumor drugs vinblastine and vincristine. These terpenoid indole alkaloids are minor products in plants, making their isolation elaborate and costly. On the

other hand, the complexity of the biosynthetic pathway of these alkaloids made it an intellectual challenge to resolve. During the study of the metabolic pathway of terpen indole alkaloids (TIA) in hairy root of *C. rouses*, MeJA was used as elicitor (Vázquez-Flota *et al.*, 1994). MeJA induces the expression of genes encoding Octadecanoid- Responsive Catharanthus AP2-domain (ORCA1, ORCA2 and ORCA3) transcription factors (Menke *et al.*, 1999; Van der Fits & Memelink, 2000) which regulate multiple genes involved in primary and secondary metabolism, including several TIA biosynthetic genes (Menke *et al.*, 1999; Van der Fits & Memelink, 2000; Van der Fits & Memelink, 2001; Aerts *et al.*, 1994). Recent results reveal the differential induction of accumulation and secretion of specific TIAs in *C. roseus* hairy roots by MeJA treatment (Ruíz-May *et al.*, 2008). Likewise, cultures of ginseng hairy roots accumulated the highest levels of ginsenoiside when MeJA was added during the progressive deceleration growth phase (Palazon *et al.*, 2003). So, the treatment of ginseng hairy roots with MeJA was used in the generation of expressed sequence tags (EST) which are useful resources for determining which genes encode enzymes that are limiting in the ginsenoide biosynthetic pathway (Choi *et al.*, 2005). Phenylalanine ammonia lyase (PAL; EC 4.3.1.5) and chalcone synthase (CHS, EC 2.3.1.74) which are key enzymes in the phenylpropanoid pathway were also induced by JAs (Dittrich & Kutchan, 1991; Hahlbrock & Scheel, 1989). Recently, putative PALs were increased in roots rice by JA treatment (Cho *et al.*, 2007). On the other hand, a protein identified as caffeic acid 3-*O*-methyltransferase was decreased in roots by JA treatment, this protein is 100% identical to quercetin 3-*O*-methyltransferase 1 that methylates OH residues of flavonoid compounds in phenylpropanoid pathway (Kim *et al.*, 2006). It should be noted that JA was reported to induce the accumulation of sakuranetin, a major phytoalexin in JA-treated rice, which is produced from methylation of naringenin by naringenin 7-*O*-methyltransferase (NOMT) with substrate specificity (Rakwal *et al.*, 1996; Kim *et al.*, 2006). Previously suggest that JAs could act as signal

mediator in the induction of the accumulation of secondary metabolites in plant roots. Recent results reveal the differential induction of accumulation and secretion of specific TIAs in *C. roseus* hairy roots by MeJA treatment (Ruíz-May *et al.*, 2008).

1.5.4. PLANT ROOTS DEFENSE SYSTEM

Plant root system constantly thrives in a diverse, everchanging environment with bacteria, fungi, viruses and other microorganisms feeding on an array of organic material (Ryan *et al.*, 2001; Rasmann & Agrawal, 2008). Compared to infection by foliar pathogens, there are many important differences in the ecology, epidemiology, life cycles, pathogenesis, and infection caused by root pathogens (Okubara & Paulitz, 2005). Plant root overcome the pathogens attack using a variety of mechanisms that include the trigger of host defense response, production of antimicrobial metabolites, inactivation of pathogen-derived toxins, lytic enzymes, and the production of a large number of pathogenesis-related proteins (PR). Most PRs and related proteins have antimicrobial activities *in vitro* through hydrolytic activities on cell walls, contact toxicity (Van Loon & Van Strien, 1999; Van Loon *et al.*, 2006) and many of then are believed to be crucial components of the plant's self-defense mechanism (Jwa *et al.*, 2006). So far, only a small number of defense pathways and resistance mechanisms have been described for leaf-pathogen interactions have been reported in roots. In response to challenge by necrotrophic fungal pathogens, roots typically exhibit the JA and ethylene dependent defense pathway (Devoto *et al.*, 2005; Turner *et al.*, 2002), but not genotype-specific resistance or the SA-dependent defense response. Although it is possible that roots lack effectors for triggering the SA pathway during invasion by necrotrophs, the absence of strong resistance might reflect the limited number of root–pathogen interactions that have been examined, and the difficulty in identifying genotype-specific interactions in below-ground pathosystems. The action of JA confers a moderate degree of host tolerance to necrotrophic root pathogens, and

plants that are compromised in JA biosynthesis or signaling show enhanced disease symptoms. The *A. thaliana fad* triple mutant (*fad3-2 fad7-2 fad8*), deficient in biosynthesis of the JA precursor linolenic acid, is more susceptible to the root pathogen *Pythium mastophorum*; 90% of the *fad* plants showed disease symptoms, as compared to about 10% of wild-type plants (Vijayan *et al.*, 1998). This susceptibility is correlated with the inability to accumulate LOX2 and PDF1.2 mRNA, indicating that a functional JA pathway is required to promote the expression of these defense-related genes in roots. Roots of *fad* plants also harbor significantly more oospores of *Pythium*. Exogenously applied MeJA reduced the level of infection in *fad* roots, but did not affect the growth rate of the pathogen *in vitro*. Another class of *A. thaliana* JA mutations, designated *jar1* (jasmonic-acid resistant), shows reduced sensitivity to jasmonate and deficient JA signaling (Staswick *et al.*, 1998). Both the *fad* and *jar1* plants exhibit enhanced susceptibility to *Pythium irregulare*.

The PR10 class of proteins were first identified as a major pollen allergen (Bet v1) from white birch (Breiteneder *et al.*, 1989). PR 10 is markedly induced by pathogen attack and plays an important role during the disease resistance response in a wide variety of plant species (Somssich *et al.*, 1986; Lo *et al.*, 1999; Midoh & Iwata, 1996). A rice PR10 protein was first characterized as a probenazole-inducible protein and initially named PBZ1 in rice (Midoh & Iwata, 1996). Interestingly, RSOsPR10 novel rice PR10 protein, is rapidly induced in roots by salt, drought stresses and blast fungus infection possibly through activation of the JA signaling pathway (Midoh & Iwata, 1996). Recently, with a proteomics approach it was possible to identify a PBZ (PR10) up-regulated by JA in rice roots (Cho *et al.*, 2007). So far, a different type of rice PR10 gene was also identified, which was shown to be up-regulated by jasmonic acid and by pathogen infection (Jwa *et al.*, 2006). Lypoxigenase 2, Chitinase, type-1 PR, translationally controlled tumor protein, polyketide synthase type I and germin protein 4 are also up-

regulated by JA and pathogenic attack in roots (Cho *et al.*, 2007). Taking together, these results, is possible suggest that JAs are key components of intracellular signalling in response to pathogenic attacks in plant roost (Miersch *et al.*, 2008; Truman *et al.*, 2007; Wasternack, 2007b; Ryan, 1992).

1.5.5. ROOT EXUDATION

Roots have the remarkable ability to secrete a vast array of compounds into the rhizosphere in response to biotic and abiotic stresses in a process termed rhizosecretion (Bais *et al.*, 2001; Bais *et al.*, 2004; Walker *et al.*, 2003b). Plants produce a compositionally diverse array of more than 100 000 different low molecular weight compounds (Bais *et al.*, 2004). The plant-borne compounds around roots are extremely heterogenous in their origin and composition (Merbach *et al.*, 1999). The rhizosphere is in part regulated by the root system itself through chemicals exuded into the surrounding soil. It is estimated that nearly 5-21% of photosynthetically fixed carbon is eventually transferred to the rhizosphere in the form of root exudates (Marschner, 1995). So that root exudation clearly represents a significant carbon cost to the plants, the mechanisms and regulatory processes controlling root secretion are just now beginning to be examined (Walker *et al.*, 2003b). Root exudates include low molecular weight phytochemicals as well as high molecular weight compounds (Walker *et al.*, 2003a). These chemical diversity of exudates could be dramatically enhanced by elicitation process (Gleba *et al.*, 1999; Loyola-Vargas *et al.*, 2007a). Roots of *Arabidopsis thaliana* elicited with JA and other elicitors induce the secretion of a broad range of phytochemicals (Walker *et al.*, 2003b). Several of these compounds exhibited a wide range of antimicrobial activity against both soil-borne bacteria and fungi at the concentration detected in the root exudates. Legumen roots exude genistein and other isoflavonoids into the soil in order to stimulate the expression of nod-genes in *Rhizobium* and *bradyrhizobium sp*

(Hungria *et al.*, 1991). The secretion of geniestein from roots of yellow lupine (*Lupines luteus*) was stimulated dramatically by MeJA applied at concentration of 100 µM (Kneer *et al.*, 1999). The differential exudation of several secondary metabolites in hairy roots of *C. roseus* by MeJA was also observed. The presence of serpentine ajmaline and catharanthine in the exudates of the hairy roots was detected only in the samples treated with MeJA (Ruíz-May *et al.*, 2008). It was previously suggested that the MeJA is involved in the mechanisms which regulate the secretion process of TIAs. Recent results from Eichhorn et al. (Eichhorn *et al.*, 2006) and Badri et al. (Badri *et al.*, 2008) suggest that transport systems seem to be involved in the root exudation of phytochemicals, in response to the MeJA treatment.

Proteins are among the high molecular weight compounds exudated by roots. Roots exudates a battery of pathogenesis-related (PRP) proteins such as β-1,3-glucanase, chitinase, and protease which are suggested to defend the plant against potential soil-borne pathogens(Bais *et al.*, 2004). Plant roots have evolved a range of mechanisms for increasing the availability of phosphorous (P), including exudation of organic acids and enzymes, particularly acid phosphatases (Raghothama, 1999). Acid phosphatases (APaes) are the most thoroughly understood plant root protein exudates (Raghothama, 1999; Tomscha *et al.*, 2004). The total protein secreted by roots to the rhizosphere is named secretome. The secretome could be changed by several elicitors (Oh *et al.*, 2005; Park *et al.*, 2002a). In order to identify secreted proteins involved in the pathogen response Oh et al (2005) analyzed the change in the *A thaliana* secretome in response to SA (Oh *et al.*, 2005), a plant hormone that regulates defense signaling (Durner *et al.*, 1997). Among eight identified SA induced proteins, four shared sequences with known proteins. Interestingly, they share the domain structure of plant lectins and are upregulated by several low molecular mass regulators, such as JA, and ethylene, or by altered environmental

conditions, such as salt stress (Zhang *et al.*, 2000). In addition, *C. roseus* hairy roots increased the secretion of total of proteins in the first hour of treatment. Several of them were differential secreted (E. Ruiz-May, manuscript in preparation). However, the mechanism by which proteins are secreted into the apoplasm/rhizosphere is not completely understood. It has been proposed that proteins are actively secreted from root epidermal cells (Flores *et al.*, 1999; Park *et al.*, 2002a). Several studies have suggested the possibility of vesicular trafficking and fusion as a cellular mechanism responsible for exudation (Walker *et al.*, 2003a). Recombinant proteins fused to the ER-targeting signal peptide were preferentially translocated to the cell wall and extracellular space (apoplast), and subsequently secreted from the root cells (Borisjuk *et al.*, 1999). These results indicate that the ER secretory pathway is closely linked with the root secretory pathway. The involvement of membrane transporters such as the ATP-binding cassette (ABC) transporter could provide an alternative to vesicular trafficking (Jasinski *et al.*, 2001). So far, the direct effect of JAs in the protein secretion of roots to the rhizosphera is unknown, but JAs have a great potential to regulate the mechanism of protein secretion considering the presence of several pathogenesis related proteins in the secretome that were induced in the intracellular space of the roots by this growth regulator.

1.6. HAIRY ROOTS AS STUDY MODEL

Hairy roots disease is caused by the infection of wounded higher plants with *Agrobacterium rhizogenes*. This gram negative soil bacterium transfers a DNA segment (TDNA) from its large root-inducing (Ri) plasmid into the genome of the infected plant. This T-DNA carries a set of genes that encode enzymes which control auxin and cytokinin biosynthesis. The new hormonal balance induces the formation of proliferating roots, called hairy roots that emerge at the wounding site. The hairy root phenotype is characterized by fast hormone-

independent growth, lack of geotropism and lateral branching. The genetic and biochemical stability of these differentiated cultures and their efficient productivity offer substantial advantages over cell suspensions (Guillon *et al.*, 2006). For these characteristics, hairy root cultures have been investigated for several decades for potential to produce the valuable metabolites that are present in wild type roots (Giri *et al.*, 1997; Flores *et al.*, 1999; Shanks & Morgan, 1999; Sevon *et al.*, 1992). Furthermore, the biosynthesis of several secondary metabolites are differentially regulated under rigid organ, tissue, development and environment specific control (St-Pierre *et al.*, 1999). For instance, vindoline and dimeric alkaloids are restricted to leaves and stems, whereas catharanthine is distributed equally throughout the aboveground and underground tissues (Westekemper *et al.*, 1980; Deus-Neumann *et al.*, 1987). For this reason, several decades hairy roots have been investigated as a biological system for the production of valuable compounds from medicinal plants (Guillon *et al.*, 2006). *C. roseus* is not the exception, the present interest for this plant is due to the fact that is an important source of chemotherapeutic agents with activity against several kinds of cancer (Loyola-Vargas *et al.*, 2007b). Among the more important alkaloids produced by *C. roseus* are included the vinblastine, used in treatment of Hodgskin's disease and the vincrsitine used in the treatment of leukemias (Schmeller & Wink, 1998). This plant also produces antihypertensive agents such as ajmalicine and serpentine (Shanks *et al.*, 1998). Elicitation, precursor feeding, cell permeabilization and trapping of the molecules released in the liquid medium are some strategic tools which was helping in the study of the biosynthetic pathway of secondary metabolites in *C. roseus* (Loyola-Vargas *et al.*, 2007b). The secondary plant messenger MeJA have been used as efficient elicitor of secondary metabolism of hairy roots (Vázquez-Flota *et al.*, 1994; Palazon *et al.*, 2003; Komaraiah *et al.*, 2001; Choi *et al.*, 2005). Elicitation with MeJA also enhances the secretion of metabolites into the medium (Ruíz-May *et al.*, 2008). Differential secretion of serpentine,

ajmaline, ajmalicine and catharanthine were also observed in *C. roseus* hairy roots treated with MeJA (Ruíz-May *et al.*, 2008). *Oxalis tuberosa* hairy roots released large amounts of harmine and harmaline into the medium after treatment with *Phytophthora cinnamoni* elicitors (Bais *et al.*, 2003). Likewise, various elicitors and permeabilisant agents (e.g. detergents, calcium chelators, pH, sonication, temperature and oxygen stresses) have been shown to stimulate the secretion of secondary metabolites such as betalaine from red beet hairy roots (Guillon *et al.*, 2006).

Understanding the molecular mechanisms involved in the transfer of T-DNA into plants have been lead the integration of new genes into hairy roots (Tzfira *et al.*, 2004). Integration of genes that encode enzymes of a given plant biosynthetic pathway between the T-borders of the Ri plasmid, then ransferring this construct into plant genome have also observed (Moyano *et al.*, 1999; Palazon *et al.*, 2003). Such a strategy was applied successfully to improve alkaloid production, first in *C. roseus* hairy roots and then in the hairy roots of various Solanaceae (Moyano *et al.*, 1999; Palazon *et al.*, 2003). However, the knowledge of the TIAs byosinthesis from *C.roseus* is still fragmented (De Luca *et al.*, 1998). The biosynthesis of several TIAs and several intermediates are still far from understanding (Magnotta *et al.*, 2006). The integration of new technologies like proteomics (Jacobs *et al.*, 2005) will help to elucidate the biosynthetic pathway of the major intermediates in TIAs biosynthesis. In the recent past years, hairy root technology has been significantly improved in different fields (Guillon *et al.*, 2006). Hairy roots possess a tremendous potential for phytoremediation, a process that can eliminate contaminants from industrial effluents (Guillon *et al.*, 2006). Recent progress in the scaling-up of hairy roots cultures is making this system an attractive toll for industrial processes (Hilton & Rhodes, 1990; Kwok & Doran, 1995; Rodríguez-Mendiola *et al.*, 1991; Ten Hoopen *et al.*, 1994; Williams & Doran, 2000).

1.7. FUTURE PERSPECTIVE

We could suggest that JA is among the growth regulators that play a prominent role in controlling various aspects of plant growth and development including their responses to the abiotic and biotic stresses. At present, it is largely unknown what are the molecular players regulating JA production in response to stress signals in roots. In contrast, the mechanisms whereby JA signaling triggers gene expression are beginning to be documented. In spite of its importance, the role of JA in roots is poorly characterized at a global level on proteins and genes. Recently significant differences were found in the signaling compound-elicited expression profiles of genes in roots *vs* those in leaves (Badri *et al.*, 2008). These differences could be correlated to the underground nature of roots and their exposure to higher microbial inoculum rates under natural conditions. However, the study of the mechanisms that drives and regulates the root exudation is one of the areas poorly understood and the involvement of JAs is unknown so far. Recent evidence suggests that root exudation is an active, ATP-dependent process in *A. thaliana* (Loyola-Vargas *et al.*, 2007a) and soybean (Sugiyama *et al.*, 2007). The active nature of root exudation suggests that root exudates do not represent a passive loss of carbon to the rhizosphere, as is commonly suggested, but that they play an active role in shaping the soil pathogen community. *A. thaliana* is known to accumulate and exudates the ubiquitous phenylpropanoids (Bednarek *et al.*, 2005), as well as more phylogenetically restricted glucosinolates, in its roots (Narasimhan *et al.*, 2003), and many of these compounds and others are biosynthetically up regulated by JAs (Kneer *et al.*, 1999). More attention is needed to focus on the potential implication of JAs in root processes including the exudation mechanism underlying the metabolome and proteome level. Identification and characterization of extracellular secondary metabolites and proteins provides an important means for increasing our understanding of the

physiological and molecular basis of plant resistance to environmental stress.

1.8. JUSTIFICATION

Methyl jasmonate (MeJA) is involved in several plant processes, but the great interest for this compound resides in its used as an efficient inductor for secondary metabolism in plant cultures. This growth regulator has been used for the induction of terpene indole alkaloids (TIAs) production in *C. roseus*. Because of the vast array of alkaloids produced by this plant, it is currently considered a study model for secondary metabolism. However, there are still a number of important enzymes that need to be characterized in the metabolic steps involved in the synthesis of the most important alkaloids of *C. roseus*. The efficient productivity, genetic and biochemical stability of hairy roots give one alternative for the study of secondary metabolites. Interestingly, the specific effect of MeJA on these cultures at the molecular level is unknown. Moreover, the study of molecular events where MeJA is involved have markedly focused on the aerial part of plants, discriminating the plant roots. Several important aspects about root biology such as the interactions in the rhizosphere and the exudation of vast array of compounds have scarcely studied. Therefore, the involvement of MeJA in these events must be pursued. For this reason, the main goal in the present work is to identify by proteomics approach, proteins differentialy expressed in hairy roots which are correlated with the accumulation and secretion of TIAs by the induction of MeJA.

1.9. HYPOTESIS

When plants are attacked by herbivores in the aereal parts, they induce several responses such as the overproduction of TIAs and pathogenesis related proteins (PRP). Moreover, the secretion of TIAs has been observed in the thricome of *solanum* species. These responses have been found to be mediated in part by MeJA.

Because of the complexity of the rhizosphere, we could expect a more complex response in the plant roots mediated by MeJA. Induction of TIAs and PRP are also expected in the hairy roots of *C. rosues* treatetd with MeJA.

1.10. OBJECTIVES

General

- To carry out the proteome analysis of *C. roseus* hairy roots correlated with the differential accumulation and secretion of TIAs by treatment with MeJA

Specifics

- To determine the physiological effect of methyl jasmonate on *C. rosues* hairy roots and its implication in the alteration of secondary metabolism.

- To determine the protein pattern of *C. roseus* hairy roots treated with methyl jasmonate using two dimensional electrophoresis (2DE-PAGE).

- To identify differentially expressed proteins by mass spectrometry.

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Chapter 2

DIFFERENTIAL SECRETION AND ACCUMULATION OF TERPENE INDOLE ALKALOIDS IN HAIRY ROOTS OF *Catharanthus roseus* TREATED WITH METHYL JASMONATE°

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2.1. ABSTRACT

The induction of several secondary metabolites in plants is one of the most commonly observed effects after the external addition of methyl jasmonate (MeJA). After the elicitation of *Catharanthus roseus* hairy roots with different concentrations of MeJA, changes in the accumulation of alkaloids such as ajmalicine, serpentine, ajmaline and catharanthine were observed. In addition to the increased accumulation of alkaloids in the tissues, the root exudation of phytochemicals increased compared to that of the non-treated control hairy roots. Moreover, MeJA induced differential secretion of several *C. roseus* hairy root metabolites.

Key words: *Catharanthus roseus*; hairy roots; methyl jasmonate; terpene indole alkaloids; root exudates.

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2.2. INTRODUCTION

Methyl jasmonate (MeJA) and its relatives from the oxylipin family originate from the oxidation of fatty acid in the plasma membrane of the plant cell. They are involved in several plant processes, such as growth and development (Vick & Zimmerman, 1984; Feussner & Wasternack, 2002; Wasternack, 2007; Creelman & Mullet, 1997). Because of its physical properties, MeJA is considered to be a signal molecule that mediates a systemic inter-plant communication under herbivore attack (Farmer *et al.*, 2003). In order to understand the function of MeJA, its exogenous application to several plant systems has been examined (Creelman & Mullet, 1995; Badri *et al.*, 2008). The induction of several secondary metabolites is one of the most commonly observed effects. As a result, MeJA has been used as an elicitor to study the biosynthetic pathway of several secondary metabolites such as the terpene indole alkaloids (TIAs) from *Catharanthus roseus* (Vázquez-Flota *et al.*, 1994; Aerts *et al.*, 1994; Gantet *et al.*, 1998; Lee-Parsons & Ertük, 2005). MeJA induces the accumulation of TIAs by turning on the transcription of several genes involved in their biosynthesis (Lee-Parsons *et al.*, 2004; Van der Fits *et al.*, 2000). This induction includes the intervention of early MeJA-responsive transcriptional regulators of the Orcas' transcription factors (Menke *et al.*, 1999; Van der Fits & Memelink, 2000). It has been demonstrated that exogenous MeJA can be transported easily in the phloem (Zhang & Baldwin, 1997), and is also found in the medium of cultured plant cells (Parchmann *et al.*, 1997).

The production of TIAs is cellular, tissue and organ specific (De Luca & St Pierre, 2000). Significant amounts of them are produced, stored and secreted by roots into the rhizosphere (Loyola-Vargas *et al.*, 2007a). These secondary metabolites exuded by the roots play a major role in determining different interactions in the rhizosphere and, ultimately, plant and soil community dynamics (Bais *et al.*, 2006). Although TIAs' accumulation in the roots of *C. roseus* in response to MeJA

treatment has been well documented, no attention has been paid to the effect of MeJA on the root secretion of TIAs. The reason of this lack of information in such an important biology process is probably because of the underground roots' localization, the complexity of the rhizosphere and the few experimental systems for the study on roots. In the present work, the effect of MeJA on alkaloid secretion by hairy roots of *C. roseus* and their correlation with alkaloid accumulation in hairy roots was evaluated.

2.3. MATERIAL AND METHODS

2.3.1. PLANT CULTURE

The J1 line of *C. roseus* hairy roots was obtained by infection of roots with *Agrobacterium rhizogenes* strain 1855 pBI 121.1 (Ciau-Uitz *et al.*, 1994), maintained in half strength B_5 medium (Gamborg *et al.*, 1968) supplemented with 3% (w/v) sucrose, pH 5.7 and subcultured into 250 mL Erlenmeyer flasks containing 100 mL of medium. The initial inoculum was 0.5 g. Flasks were incubated in the dark on a rotating shaker at 100 rpm at 25°C for 15 d.

2.3.2. HAIRY ROOTS ELICITATION WITH MEJA

MeJA solutions were prepared by diluting the MeJA stock (Aldrich, 95+% purity) in absolute ethanol (J. T. Baker, 99.8%). Different concentrations of MeJA (10, 100 and 250 µM) were added to 15-day-old hairy root culture and for the control treatment absolute ethanol alone was used. Root tissue and culture media were collected at 12, 24, 48 and 72 h after elicitation.

2.3.3. FRESH (FW) AND DRY WEIGHT (DW) DETERMINATION

After 12, 24, 48 and 72 h of elicitation with 10, 100 and 250 µM of MeJA, hairy roots were collected and weighed for fresh weight determination. For dry weight determination, the roots were frozen at -75°C and freeze-dried. After total elimination of water was achieved, the lyophilized roots were weighed. Each sample was done in triplicate.

2.3.4. HAIRY ROOTS ALKALOID EXTRACTION

The alkaloids were extracted as described by Monforte-González et al. (1992). Briefly, freeze-dried root tissue (0.5 g) was homogenized in 5 mL methanol (Matsumoto *et al.*, 1982). The homogenates were incubated in a water bath at 50°C for two hours. The methanolic extracts were dried under vacuum. The residue was resuspended in 3 mL of 2.5% sulphuric acid (v/v) and washed three times with 20 mL of ethyl acetate. The pH of the aqueous solution was adjusted to 9.5 with concentrated ammonium hydroxide (28%) and extracted with 10 mL of ethyl acetate three times. The organic phase was concentrated under vacuum and resuspended with 600 µL of methanol (Fisher Scientific Co.) and stored at -20°C for alkaloid analysis.

2.3.4. PHYTOCHEMICALS MEDIUM EXTRACTION

To examine the secreted phytochemicals from *C. roseus* hairy roots, liquid media samples from *in vitro*-grown *Catharanthus* hairy roots were collected from one flask (final volume of 100 mL), filtered through a nylon syringe filter of pore size 0.40 µm (Life Sciences Cat. PN 4612 or Nalgene cat. 195-2520) to remove any cellular debris, and concentrated by freeze-drying (Labconco) to remove water. The concentrate was dissolved in 5 mL 2.5% (v/v) sulphuric acid and extracted as described for root biomass. The final concentrate was dissolved in 500 µL of absolute methanol (Fisher Scientific Co.) and analyzed by HPLC. The same procedure was followed for each treatment.

2.3.5. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) ANALYSIS OF ALKALOIDS FROM THE ROOTS AND THE EXUDATES

Compounds from roots and media were chromatographed by gradient elution on a 4.6 mm x 150 mm reverse phase, Zorbax Eclipse XDB, 5-µm particle size C_{18} column (Agilent technology). The chromatographic system (Agilent series 1100) consists of two G1312A pumps connected to a G1328A manual sample injector. The injected samples (20 µl) were detected at 280 nm with a variable UV-vis detector G1365B (Agilent technology). The mobile phase consisted of acetonitrile: 0.01M $(NH_4)_2HPO_4$ (52:48) a flow rate of 1.5 mL min^{-1}. The solution was filtered through a 0.22 µm nylon filter and degassed under vacuum.

Retention times and peak heights of commercially purchased ajmaline, serpentine, vincamine, vindoline, ajmalicine and catharanthine (Sigma Chemical Co.) and previously identified in *Catharanthus* hairy root cultures (Loyola-Vargas *et al.*, 2007b) were used to determine a compound's possible presence in root exudates and to calculate the concentrations of these compounds in root tissues and exudates.

Statistical analysis. At least three replicates of all experiments were conducted. The statistical analysis was performed by one-way ANOVA analysis, taking $P \leq 0.05$ and $P \leq 0.01$ (Tukey's test) as significant and highly significant, respectively.

2.4. RESULTS

2.4.1. TOTAL ALKALOID ACCUMULATION AND SECRETION IN HAIRY ROOTS TREATED WITH MEJA.

It is well-known that different secondary metabolites accumulate in response to increasing concentrations of MeJA (Spollansky *et al.*, 2000; Colque *et al.*, 2004; Lee-Parsons *et al.*, 2004). However, little attention has been paid to the secretion of these

compounds in response to this elicitor. In this study, the effect of MeJA concentration (0, 10, 100 and 250 μM) and exposure time on TIAs production and secretion were investigated during a temporal course of 72 h using hairy roots of *C. roseus*.

After 15 days of cultures, which correspond to the exponential phase of the culture cycle (Ciau-Uitz *et al.*, 1994), the root cultures were changed to fresh medium contained 50 μL of ethanol (control) or 50 μL of each of the different MeJA concentrations. In our study, the increase in the concentration of MeJA caused an increment in the levels of total alkaloids throughout the temporal course of 12, 24, 48 and 72 h. The highest accumulation of alkaloids was observed after 48 h, when 100 and 250 μM of MeJA was added (Fig. 2.1A); in fact, both treatments followed a very similar development, after 48 h of induction, the amount of the total alkaloids increased more than three times, observing high significant difference with 250 μM MeJA. In the case of the secretion of alkaloids into the culture medium, the highest level of total alkaloid exudation was observed after 12 h of treatment (Fig. 2.1B). After alkaloid secretion reached a maximum, it followed the continuous decline of the alkaloids' presence in the culture medium (Fig. 2.1B). In the case of the control, the relationship between the amount of alkaloids in the roots and the medium was 1.40 after 12 h. In the case of the treatment with 250 μM of MeJA, this relationship was only 1.16 after 12 h of elicitation. This relationship changed to 1.25 after 48 h for the control and to 4.31 for the samples treated with 250 μM of MeJA. In other words, the secretion of the alkaloids was higher during the first 12 h of the treatment. The change in the amount of alkaloids correlates with the decrease in the DW of the hairy roots (Fig. 2.2). High significant reduction in the DW was observed in the presence of 250 μM of MeJA after 12 h of treatment. The DW of the hairy roots treated with 250 μM of MeJA decreased 33% in comparison with the control. The other two treatments did not change the DW of the hairy root cultures.

2.4.2. TIAS ACCUMULATION PATTERNS IN HAIRY ROOTS TREATED WITH MEJA.

In the present study we analyzed the accumulation pattern of four individual alkaloids already identified in *C. roseus* hairy roots: ajmalicine, serpentine, ajmaline and catharanthine (Vázquez-Flota *et al.*, 1994). The amount of ajmalicine increased significantly as the dosage of MeJA increased along the time course (Fig. 2.3A). The highest level of ajmalicine accumulation was observed with the treatments of both 100 and 250 μM MeJA. Ajmalicine increased from 2.90 mg g^{-1} DW in the control to 5.73 and 6.34 mg g^{-1} DW with the treatments of 100 and 250 μM MeJA, respectively after 72 h (Fig. 2.3A).

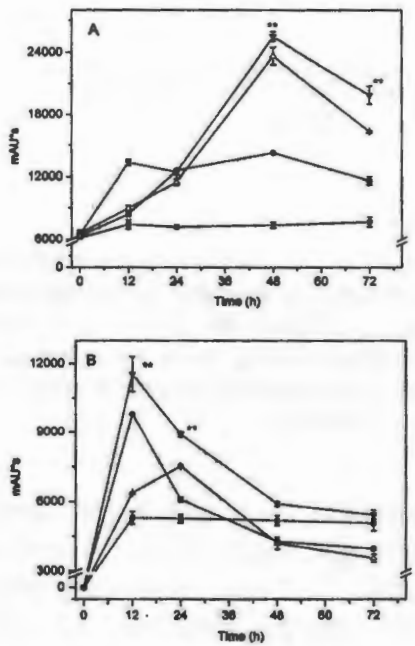

Figure 2.1. *Total alkaloid accumulation (A) and secretion (B) in hairy roots of C. roseus elicited with MeJA (■, 0 μM; ●, 10 μM; △, 100 μM; and ▼, 250 μM). Average of three replications plotted. Error bars represent ± SE (n=3). Asterisk represent statistical significance of mean differences at a given time by Turke´s test (*, P≤0.05; **, P≤0.01).*

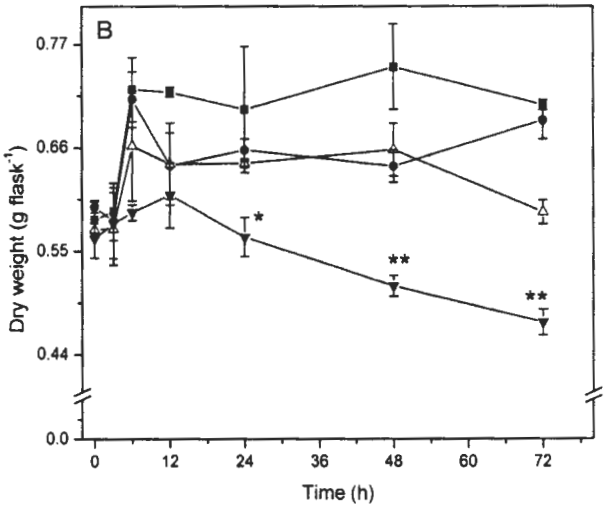

Figure 2.2. *Influence of MeJA on dry weight of hairy roots through 72 h of induction. Hairy roots were treated as indicated in "materials and methods", in the absence (■) or presence of MeJA (●, 10 μM; △, 100 μM; and ▼, 250 μM). Average of three replications plotted. Error bars represent ± SE (n=3). Asterisk represent statistical significance of mean differences at a given time by Turke´s test (*, P≤0.05; **, P≤0.01).*

The serpentine accumulation level was significantly increased with the treatment of 250 μM MeJA. In the control, the level of serpentine was 0.58 mg g^{-1} DW and increased three times until reach 1.71 mg g^{-1} DW with the treatment of 250 μM MeJA throughout the elicitation time course (Fig. 2.3B). A similar level of serpentine was observed after 72 h of induction with 10 μM MeJA (Fig. 2.3B).

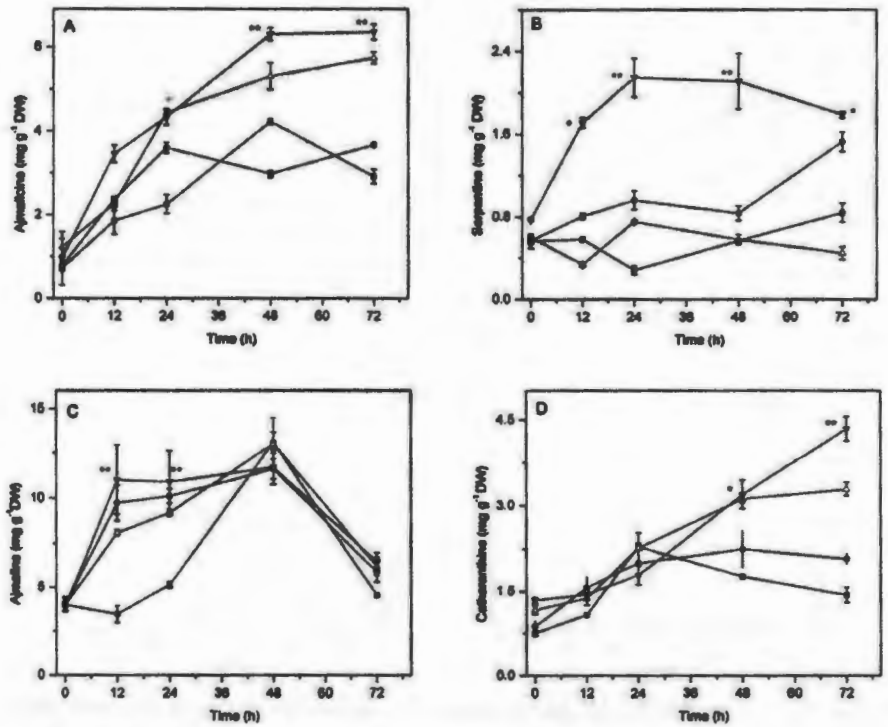

Figure 2.3. *Ajmalicine (A), serpentine (B), Ajmaline (C) and catharanthine (D) production in hairy roots of C. roseus elicited with MeJA (■, 0 μM; ●, 10 μM; △, 100 μM; and ▼, 250 μM). Average of three replications plotted. See legend to Fig. 2.2 for details.*

The ajmaline accumulation was also modified by the MeJA treatment (Fig. 2.3C). The three treatments produced a highly significant increase in the amount of ajmaline during the first 24 h, reaching levels of around 10 mg g^{-1} DW (Fig. 2.3C). After 48 h of treatment, the accumulation of ajmaline was similar in the control as well as the treatments which reached the amount of 12 mg g^{-1} DW. Then, the concentration of ajmaline dropped drastically to 7 mg g^{-1} DW for both the control and the treatments (Fig. 2.3C).

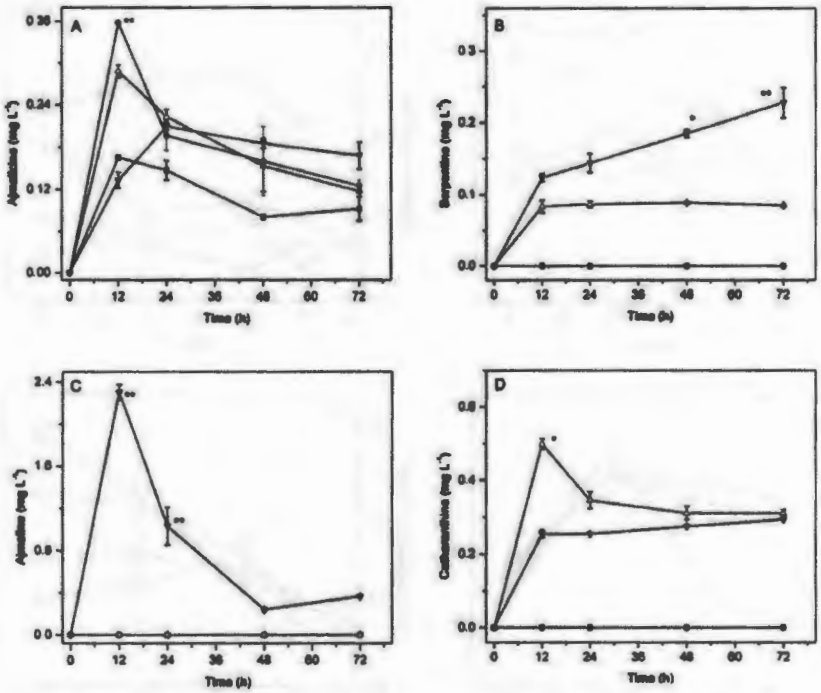

Figure 2.4. *Ajmalicine (A), serpentine (B), Ajmaline (C) and catharanthine (D) excretion in hairy roots of C. roseus elicited with MeJA (■, 0 μM; ●, 10 μM; △, 100 μM; and ▼, 250 μM). See legend to Fig 2.2 for details.*

The accumulation pattern of catharanthine in *C. roseus* hairy roots throughout the temporal course showed a continuous increase with treatments of 100 and 250 μM of MeJA (Fig. 2.3D). The accumulation of catharanthine detected after 72 h of treatment rose significantly threefold, from 1.42 mg g^{-1} DW in the control to 4.34 mg g^{-1} DW with 250 μM MeJA treatment.

2.4.3. TIAS EXUDATION PATTERNS IN HAIRY ROOTS TREATED WITH MEJA.

The secretion of ajmalicine increased significantly in the first 12 h of treatment (Fig. 2.4A), from 0.165 mg L^{-1} in the control to 0.358 mg L^{-1} in the treatment with 10 μM MeJA, more than two

times the amount of ajmalicine. After the first 12 hours the secretion of ajmalicine decreased steadily until the end of the temporal course to levels of 0.15 mg L^{-1}.

The secretion of serpentine was detected only in the treatments of 100 and 250 µM MeJA (Fig. 2.4B); neither the control nor the treatment of the hairy root cultures with 10 µM MeJA displayed secretion of serpentine into the culture medium. However, serpentine secretion increased from zero in the medium of the control to 0.227 mg L^{-1} of medium with the 250 µM MeJA treatment after 72 h. The level of secretion of serpentine with treatment of 100 µM MeJA was continuously maintained around 0.088 mg L^{-1}. High significant difference was observed at the last 24 h of treatment with 250 µM MeJA when it is compared with the treatment of 100 µM MeJA. The secretion of ajmaline was detected exclusively after the treatment with 250 µM MeJA (Fig. 2.4C). After 12 h of treatment, the accumulation of ajmaline in the culture medium reached 2.30 mg L^{-1}. After this point the level of ajmaline decreased continuously to 0.367 mg L^{-1} after 72 h of treatment.

The secretion of catharanthine was detected exclusively in treatments with 100 and 250 µM MeJA (Fig. 2.4D). The treatment with 100 µM MeJA produced a peak in secretion, 0.49 mg L^{-1} of catharanthine, after 12 h of induction, after which the amount of catharanthine decreased to 0.3 mg L^{-1}. The amount of catharanthine secreted by the hairy roots treated with 250 µM MeJA reached a maximum at 12 h with 0.27 mg L^{-1}. At this time the treatment with 100 µM MeJA showed significant difference in compare to the treatment with 250 µM MeJA.

2.5. DISCUSSION

MeJA and jasmonic acid (JA) are very powerful elicitors of secondary metabolism in plants (Zhao *et al.*, 2005). It has been determined that these growth regulators can induce an increase in the concentration of Paclitaxel and baccatin III in free and immobilized cells of *Taxus baccata* (Bonfill *et al.*, 2007),

phenylpropanoids and naphtodianthrones in cell suspensions of *Hypericum perforatum* L. (Gadzovska *et al.*, 2007), ajmalicine in *C. roseus* cell cultures (Lee-Parsons *et al.*, 2004) and nicotine in *Nicotiana attenuata* (Baldwin, 1996), among others.

It is a well known fact that MeJA affect the growth of roots (Staswick *et al.*, 1992) and suspension cultures (Goossens *et al.*, 2003). In our study, the DW of hairy roots decreased significantly when they were treated with 250 μM MeJA (Fig. 2.2). However, the best induction and secretion of the four studied alkaloids were carried out with the addition of 250 μM MeJA. If we add the alkaloid content from each flask and each treatment through the temporal course it can be seen how the accumulation of alkaloids increases as a function of the dosage of MeJA (Fig. 2.5). Serpentine, by far, is the most responsive alkaloid to elicitation (Fig. 2.5B).

Our results for the accumulation of alkaloids into the tissues are similar to those previously reported for suspension cultures, seedlings and hairy root cultures elicited with MeJA (Vázquez-Flota *et al.*, 1994; Aerts *et al.*, 1994; Gantet *et al.*, 1998; Lee-Parsons & Ertük, 2005; Lee-Parsons *et al.*, 2004). Vazquez-Flota et al. (1994) induced hairy roots of *C. roseus* with 100 μM MeJA and got a similar induction pattern for ajmalicine and catharanthine. The accumulation of both TIAs in hairy roots is directly proportional to the dosage of MeJA and the time of the induction (Figs. 2.3A and 2.3D).

The secretion of the four alkaloids studied here was significant increased after 12 h of induction (Fig. 2.4D). Lee et al. (2005) analyzed the secretion of ajmalicine in suspension cultures of *C. roseus* treated with 100 μM MeJA. The enhancement of secretion of ajmalicine was directly proportional to the concentration of MeJA and was higher in the first 12 h after the induction with 100 μM MeJA.

The presence of serpentine and catharanthine in the exudates of the hairy roots was detected only in the samples treated with 100 and 250 μM MeJA (Figs. 2.5B and 2.5D). The ajmaline was secreted only in the presence of the highest amount of MeJA (Fig. 2.5C). These results clearly show a differential secretion of

TIAs from the hairy roots treated with MeJA. The kind and the amount of TIAs secretion depend on the elicitor's concentration used in the hairy roots culture. The effect of the treatment did not change the rate of conversion of serpentine in ajmalicine by effect of MeJA as can be observed in figure 3. El-Sayed and Verpoorte (2005) found a similar pattern during the aging of leaves of *C. roseus* sprayed with 300 μM of MeJA.

Recent results from Eichhorn et al. (2006) and Badri et al. (2008) suggest that transport systems seem to be involved in the root exudation of phytochemicals, in response to the MeJA treatment. In soybean, *Gm*PDR12 is rapidly responsive to salicylic acid and MeJA (Eichhorn *et al.*, 2006). Salicylic acid is required for the execution of the hypersensitive reaction in soybean cell suspension cultures inoculated with *Pseudomonas syringae* pv. glycinea. In the other study, using *Arabidopsis thaliana*, a considerable number of transporters were found to be differentially regulated by nitric oxide, salicylic acid and MeJA treatments (Badri *et al.*, 2008). Salicylic acid and MeJA treatments showed both up- and down regulation of transporters. MeJA differentially regulated MATE, an H^+ ATPase pump, a sugar transporter and a metal transporter. Badri et al.'s (2008) results showed that only one full-length ABC transporter (AtMRP2) was significantly upregulated upon SA and MeJA treatment compared with the control. The genes *AtATH10*, *AtNAP5* and *AtTAP2* were also down regulated significantly upon MeJA treatment (Badri *et al.*, 2008). It is possible suggest that MeJA could be regulating the expression of some of the transporters involved in the secretion of the compounds produced by the *C. roseus* hairy roots. New experiments are on the way in our laboratory to enlighten this fundamental aspect about the secondary metabolism.

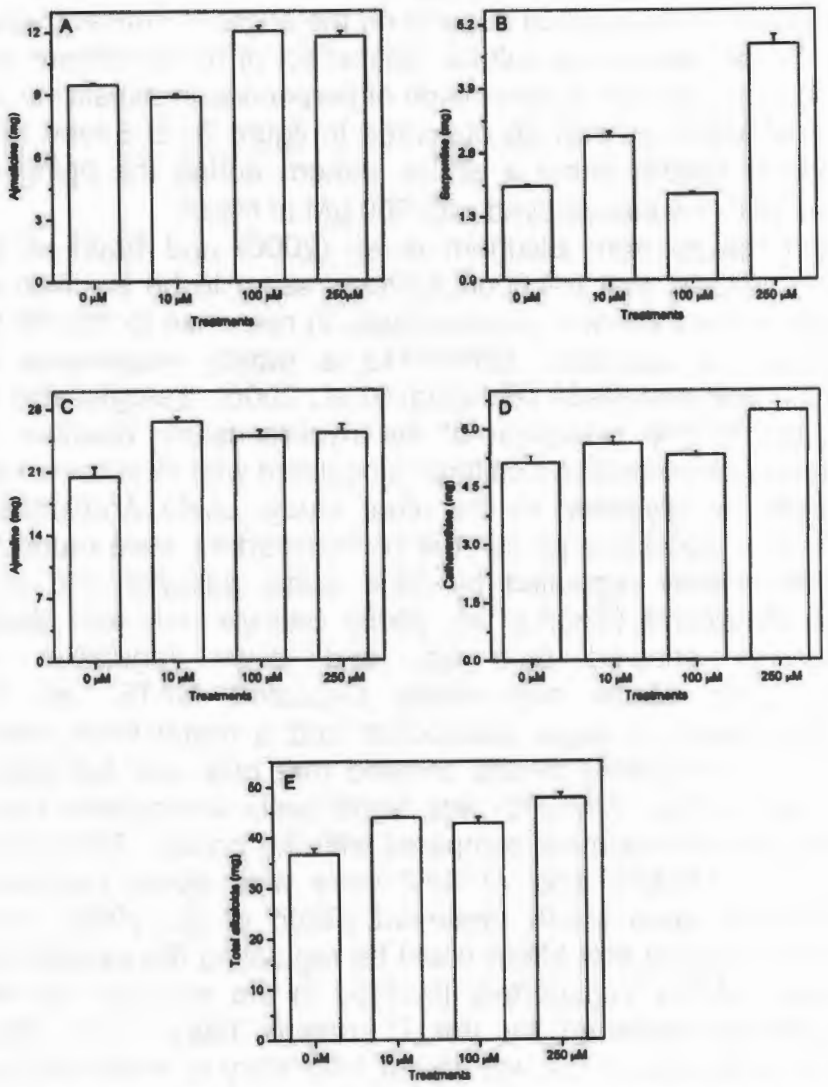

Figure 2.5. *Total alkaloids per experiment. Accumulation into the tissues and secretion from hairy roots culture throughout the temporal course with different concentrations of MeJA. (A) Total accumulation and secretion of (A) ajmalicine, (B) serpentine, (C) ajmaline, (D) catharanthine, and (E) sum of ajmalicine, serpentine, ajmaline and catharanthine. The results are the average of three independent experiments. Error bars represent ± SE.*

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Chapter 3

PROTEOMICS SURVEY OF *Catharanthus roseus* (L.) G. Don HAIRY ROOTS TREATED WITH METHYL JASMONATE°

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3.1. ABSTRACT

Jasmonates (JAs) are specific signaling molecules in plants that are involved in diverse set of physiological and developmental processes. Pathogen attack and wounding inflicted by herbivores induce the biosynthesis of these molecules, triggering defense responses both locally and systemically. However, the effects of JAs in roots have been poorly studied. Two-dimensional gel electrophoresis revealed several proteins differentially expressed in hairy roots treated with MeJA compared to the controls. Using a proteomic approach, we were able to identified 58 different proteins belonging to eleven functional categories. Proteins involved in carbohydrate metabolism (21%), cell growth and organization (10%), energy (7%) and cell cycle (3%) were highly repressed. On the other hand, amino acid metabolism (12%), protein modification and chaperons (12%) and secondary metabolites (7%), were found in abundance suggesting changes in the metabolic status of hairy roots treated with MeJA. Changes in several metabolic enzymes due to MeJA exposure could be correlated with the inhibition of hairy root growth.

Key words: *Catharanthus roseus*, methyl jasmonate, proteomics, roots, terpene indole alkaloids.

3.2. INTRODUCTION

Methyl jasmonate (MeJA) and related molecules called jasmonates (JAs) have been shown to play a crucial role orchestrating stress response in plants such as root growth inhibition, tuber formation, tendril coiling and touch, flower development and senescence (Creelman & Mullet, 1997; Aerts *et al.*, 1994; Gantet *et al.*, 1998; Rijhwani & Shanks, 1998; Lee-Parsons *et al.*, 2004; Wasternack, 2007a). Environmental stresses, such as wounding or pathogen attack, can trigger JAs production *in planta*. Upon perception of JAs signals, the plant cell activates several defense mechanisms, reflected in a massive reprogramming of gene expression (Pauwels *et al.*, 2008). Previously, an increase of JAs in cells has been correlated with fresh weight lost, protein lost and cell death in several plant systems (Creelman & Mullet, 1997; Zhang & Xing, 2008). Moreover, the effectiveness of JAs to elicit secondary metabolism in cell cultures has been used as a tool to unravel the complex cellular process of terpene indole alkaloid (TIAs) biosynthesis (Vázquez-Flota *et al.*, 1994; Palazon *et al.*, 2003). Recently, differential accumulation and secretion of TIAs in hairy roots of *C. roseus* treated with MeJA was observed (Ruíz-May *et al.*, 2008).

Microarray technology and metabolic profiling have been used to examine the role of MeJA in the regulation of secondary metabolism resulting in the establishment of gene-to-metabolite networks. These networks are involved in the biosynthesis of the pharmaceutically valuable TIAs in periwinkle (*Catharanthus roseus*) cells (Rischer *et al.*, 2006), in the characterization of enzymatic steps in the isoflavone and triterpene biosynthetic pathways in barrel medic (*Medicago truncatula*) cells (Naoumkina *et al.*, 2007; Achnine *et al.*, 2005). The best response known for JAs in higher plants correspond to the aerial parts (Seo *et al.*, 2001; Gatehouse, 2002). However, the knowledge about plant roots defense response and other root

interactions is poorly understood. The reason for this lack of information is probably because of the underground roots' localization, the complexity of the rhizosphere and the few experimental systems for the study on roots. On the other hand, in ecological perspective, below ground processes are now recognized as essential components of ecosystem productivity and stability (Van der Putten *et al.*, 2001). The surface area of roots can far exceed that of aerial parts, thus providing tremendous resources for microbes, nematodes, and arthropods in the soil. These organisms are now recognized as drivers of plant diversity and ecosystem function (De Deyn & Van der Putten, 2005).

Because of their stable expression of metabolic pathways and rapid growth, hairy roots established by *Agrobacterium rhizogenes* are one of the best alternatives for the study of root biology (Flores *et al.*, 1987; Rhodes *et al.*, 1990). Hairy roots have been used to study the biosynthesis and regulation of alkaloids, flavonoids and sesquiterpenes in medicinal plants (Jung & Tepfer, 1987; Kamada *et al.*, 1986; Rhodes *et al.*, 1986). Hairy root cultures can also be successfully utilized for the *in vitro* production of important proteins. For instance hairy root cultures of carrot produced higher levels of peroxidase (POD; EC 1.11.1.7) than the suspension cultures. Horseradish hairy root cultures have also been reported to produce considerably higher level of PODs (Uozumi *et al.*, 1992; Flocco *et al.*, 1998). Interesting MeJA induce the production of reactive oxygen species (ROS) in the plant cell where PODs have crucial role in scavenge ROS (Zhang & Xing, 2008). Moreover, jasmonate induced proteins such as ribosome-inactivating proteins (RIPs) (Reinbothe *et al.*, 1994) and has been also successfully purified from hairy roots of *Phytolacca americana* (Park *et al.*, 2002). RIPs are *N*-glycosidases that remove a specific adenine from the sarcin/ricin loop of the large rRNA, thus arresting protein synthesis at the translocation step (Endo & Tsurugi, 1987). Hairy roots metabolic engineering can also be used to produced therapeutically active foreign proteins, such as a human secreted alkaline phosphatase (SEAP) (Guillon *et*

al., 2006). Therefore, hairy toots appear to be a very convenient system for the production of proteins to be used in pharmaceutical industry (Guillon *et al.*, 2006).For these reasons, in the present work, we used a proteomics approach to identify proteins synthesized in *C. roseus* hairy roots in response to MeJA. This systematic proteomic study led to the identification of 58 proteins differentially expressed in response to MeJA which are involved in numerous cellular functions.

3.3. MATERIAL AND METHODS

3.3.1 PLANT CULTURE

The J1 line of *C. roseus* hairy roots was obtained by infection of roots with *Agrobacterium rhizogenes* strain 1855 pBI 121.1 (Ciau-Uitz *et al.*, 1994). This line was maintained in half strength B_5 medium (Gamborg *et al.*, 1968) supplemented with 3% (w/v) sucrose and subcultured in 250 mL Erlenmeyer flasks containing 100 mL of medium. The initial inoculum was 0.5 g FW. Flasks were incubated in the dark on a rotating shaker at 100 rpm at 25°C for 15 days.

3.3.2 HAIRY ROOTS ELICITATION WITH MEJA

MeJA solutions were prepared by diluting the MeJA stock (Aldrich, 95>% purity) in absolute ethanol (J. T. Baker, 99.8%). Different concentrations of MeJA (10, 100 and 250 µM) were added to 15-day-old hairy root culture and for the control treatment absolute ethanol alone was used. Root tissue and culture media were collected at 12, 24, 48 and 72 h after elicitation.

3.3.3. FRESH (FW) AND DRY WEIGHT (DW) DETERMINATION

After 12, 24, 48 and 72 h of elicitation with 10, 100 and 250 µM of MeJA, hairy roots were collected and weighed for fresh

weight determination. For dry weight determination, the roots were frozen at -80°C and freeze-dried. After total elimination of water was achieved, the lyophilized roots were weighed. Each sample was done in triplicate. The experiment was repeated twice.

3.3.4. ELECTRON MICROSCOPY

The hairy root tissues exposed to 10 and 100 µM of MeJA were sampled after 72 h of incubation. All tissues were fixed in 1% glutaraldehyde, 4% formaldehyde in 50 mM sodium phosphate buffer at pH 7.2 during 3 hours, and rinsed for 30 minutes with the same buffer. The fixed tissues were dehydrated in a graded series of 10%, 30%, 50%, 70%, 90% and 100% ethanol for 60 min each. The samples were mounted on a metallic grill (Polaron SEM coating system E S100) and plated with gold using 30 mA during 60s at 120 mTorr until a layer of 150 Á was reached. The samples were observed using a scanning electronic microscope (GEOL JSM 6360 LV). Photographs were obtained by projecting the images to angles of +8° and -8° from the optical axis.

3.3.5. SAMPLE PREPARATION FOR 2-DE PAGE

For protein extraction, a frozen whole hairy root was ground in liquid nitrogen with a mortar and pestle, which had been pre-cooled with liquid nitrogen. The resultant material was homogenized with the extraction buffer (total volume 6 mL) which consisted of 7 M urea, 2 M thiourea, 4% (v/v) NP-40, 1% (v/v) DDT, 1 mM PMSF, 10 mM EDTA, 40 mM Tris-HCl, 1% (v/v) ampholines mixture (pH 3-10) and 0.05% PVPP. The homogenates were centrifuged at 15,000 x *g* for 15 min. The supernatants were precipitated with 25 mL of cold (-20°C) 10% (w/v) TCA in acetone containing 0.07% (v/v) β-mercaptoethanol. The sample was maintained at -20°C for 2 h to complete precipitation. After centrifugation (15 min, 3,000 x *g*), the samples were washed twice with 10 mL acetone (-20°C)

containing 0.07% (v/v) β-mercaptoethanol to remove the TCA. The precipitate was lyophilized for 1 h and then solubilized in 1 mL of lysis buffer containing 7 M urea, 2 M thiourea 2% (v/v) NP-40 and 2% (v/v) ampholines mixture [1.6% (v/v) pH 5-7 and 0.4% (v/v) pH 3-10]. This mixture was vortexed thoroughly, and then centrifuged (15 min, 16,000 x *g*). The supernatant was recovered and stored in aliquots at -80°C. The protein concentration was determined by the method of Peterson (1977).

3.3.6. TWO-DIMENSIONAL ELECTROPHORESIS (2-DE) SEPARATION

Isoelectric focusing (IEF) was carried out in capillary glass tubes (15 X 1.5 mm inside diameter) sealed at the bottom with Parafilm. The gel solution mixture (total volume 3 mL) consisted of 9.5 M urea, 2% (v/v) Nonidet P-40 (NP-40), 4% (w/v) polyacrylamide and 2% (v/v) ampholines mixture [1.6% (v/v) pH 5-7 and 0.4% (v/v) pH 3-10]. The solution was degassed for 10 min, 2 µL of 10% (w/v) ammonium persulfate and 2 µL of TEMED were immediately added and the solution was loaded in the glass tubes. The gels were allowed to set for 1 h, after polymerization the parafilm was removed and the gels were then placed in the electrophoretic chamber. The top of the gels was loaded with lysis buffer (total volume 3 mL) (9.5 M urea, 2% (v/v) NP-40 and 2% (v/v) ampholines mixture [1.6% (v/v) pH 5-7 and 0.4% (v/v) pH 3-10]. The bottom reservoir of the electrophoretic chamber was filled with 0.01 M H_3PO_4 and the upper reservoir was filled with 0.03M NaOH which was extensively degassed to remove CO_2. The gels were then pre-electrophoresed at 200 V for 10 min, 300 V for 15 min and 400 V for 15 min. The power was turned off and the upper reservoir was emptied, lysis buffer was removed from the surface of the gels and the protein samples were loaded. The samples were overlaid with buffer (9 M urea, 1% (v/v) ampholytes mixture [0.8% (v/v) pH 5-7 and 0.2% (v/v) pH 3-10] and 0.05% (p/v) bromophenol blue). The gels were placed in the electrophoretic

chamber and the upper reservoir was filled with 0.03 M NaOH. The gels were isoelectrically focused at 600 V for 5 h, 800 V for 12 h and 1200 V for 5 h (O'Farrell, 1975). After focusing, the gels were incubated in equilibration buffer [0.0625 M Tris HCl, pH 6.8, 2.3% (w/v) SDS, 1% (w/v) DDT, 30% glicerol (v/v) and 0.05% (w/v) bromophenol blue] and then the gels were agitated gently at room temperature for 15 min. After equilibration, the gels were placed on the top of a resolving SDS polyacrylamide gel (12.5% T, 1.5 mm thick) and electrophoresed at 150 V for 5 h. The resultant 2-DE gels were stained overnight with Coomassie Brilliant Blue R-250 and destained the next day. Image analysis was performed visually, and the changes observed were qualitative in nature. The 2-DE PAGE gels experiments were repeated at least three times to confirm reproducibility.

3.3.7. IN-GEL TRYPSIN DIGESTION

Protein spots were manually excised from the gel, washed twice with water for 15 min, and destained with a 1:1 (v/v) solution of acetonitrile and 50 mm ammonium bicarbonate while changing solutions every 30 min until the blue color of Coomassie was removed (Watson *et al.*, 2003). These gel plugs were transferred to polypropylene 96-well plates for further processing. The gel spots were dehydrated with 25 µL of acetonitrile (ACN) each for 15 min at room temperature. After ACN removal, the gel spots were dried under vacuum and rehydrated in 20 µL of sequencing-grade modified bovine trypsin (10 ng μL^{-1} in 25 mM ammonium biocarbonate, Roche Diagnostics). After rehydration for 20 min on ice, excess trypsin solution was removed and 15 µL of 25 mM ammonium bicarbonate was added to each well to prevent dehydration during incubation. Proteolysis was allowed to continue 13 h at 37°C and stopped by adding 15 µL of 10% formic acid. Peptides were extracted sequentially with 25 mM ammonium bicarbonate:acetonitrile (1:1) and 100% acetonitrile, pooled and concentrated to a final volume of 10-15 µl.

3.3.8. LC/MS/MS

Separations of the digest's proteins were achieved using a nanoscale HPLC system (LC Packings, San Francisco, CA) consisting of an autosampler (Famos), a precolumn switching device (Switchos), and an HPLC pump system (Ultimate). Samples (5 µL) were loaded onto a C18 precolumn (0.3-mm inner diameter x 1.0 mm, 100 Å, PepMap C18, LC Packings) for desalting and concentrating at a flow rate of 50 µL min^{-1} using mobile phase A (5% ACN and 95% water containing 0.1% formic acid). The desalted peptides were then eluted from the precolumn and separated on a nano analytical C18 column (75-µm ID X 15 cm, 100 Å, PepMap C18, LC Packings) at a flow rate of 200 nL min^{-1}. Peptides were eluted with a linear gradient of 5 – 40% mobile phase B (95% ACN and 5% water containing 0.08% formic acid) over 40 min. The separated peptides were directly analyzed with an ABI QSTAR Pulsar I hybrid Q-TOF mass spectrometer (Applied BioSystems) equipped with a nanoelectrospray ionization source (Protana). The nanoelectrospray was generated using a PicoTip needle (10-µm inner diameter, New Objectives, Woburn, MA) maintained at a voltage of 2400 V. TOF-MS and tandem mass spectral data were acquired using information-dependent acquisition (IDA) with the following settings: charge state selection from 2 to 5, an intensity threshold of 10 counts s^{-1} for tandem experiments, and a collision energy setting automatically determined by the IDA based on the m/z values of each precursor ion. Following IDA data acquisition, precursor ions were excluded for 90 s using a window of 6 amu to minimize the redundancy in tandem mass spectra.

3.3.9. DATABASE QUERIES AND PROTEIN IDENTIFICATION

For protein identification, the acquired mass spectral data were queried against the NCBI non-redundant protein database (NCBInr), downloaded on February 2007, using the MASCOT

(version 2.2, Matrix Science Ltd., London, UK) search engine (Perkins *et al.*, 1999; Creasy & Cottrell, 2002) with the following settings, a mass tolerance of 150 ppm, one trypsin missed cleavage allowance, 2+ or 3+ peptide charge, and two variable amino acid modifications, i.e., methionine oxidation and cysteine carbamidomethylation. Only protein identifications with a molecular mass search (MOWSE) score greater than the generally accepted significant threshold (determined at 95% confidence level as calculated by MASCOT; $p < 0.05$) and at least two matched peptides are reported in this study.

3.4. RESULTS

3.4.1. MEJA EFFECT ON HAIRY ROOTS GROWTH

MeJA inhibits the growth of tissues (Staswick *et al.*, 1992; Wasternack, 2007b) at the same time that it promotes different physiological responses. In our experiments, hairy roots were treated with different concentrations of MeJA (0, 10, 100 and 250 μM) for 72 h (Fig. 3.1). The reduction of fresh and DW was significantly different with 250 μM MeJA at 48 h (Fig. 3.1A) and 72 h (Fig. 3.1B) when compared to the control. At 72 h, the DW dropped from 0.705 to 0.474 g flask^{-1}. The treatments with 10 and 100 μM did not decrease the growth of the hairy roots (Fig. 3.1). Because of the severe negative effect of the highest concentration of MeJA (250 μM) on root's growth, it was decided to exclude it from the following experiments.

The visual analysis of the hairy root cultures showed no change in the phenotype of the tissues treated with 10 and 100 μM MeJA (Figs. 3.2B and 3.2C). However, observations of the tissues with the scanning electron microscope showed clear and remarkable modifications in the morphology of the root cap of C. *roseus* treated with MeJA (Figs 3.2E and 3.2F). In the control (Fig. 3.2D), the root cap had a normal morphology (Rueffer *et al.*, 1978). The cap of the tissues treated with 10 μM MeJA (Fig. 3.2E) showed a more spherical morphology than

roots from the control treatment (Fig. 3.2D). Furthermore, in the hairy roots treated with 100 μM MeJA (Fig. 3.2F), a drastic change in the root cap morphology of the hairy roots was observed. The lateral root cell layer disappears and even the epidermis is damaged by the presence of MeJA (Fig. 3.2F).

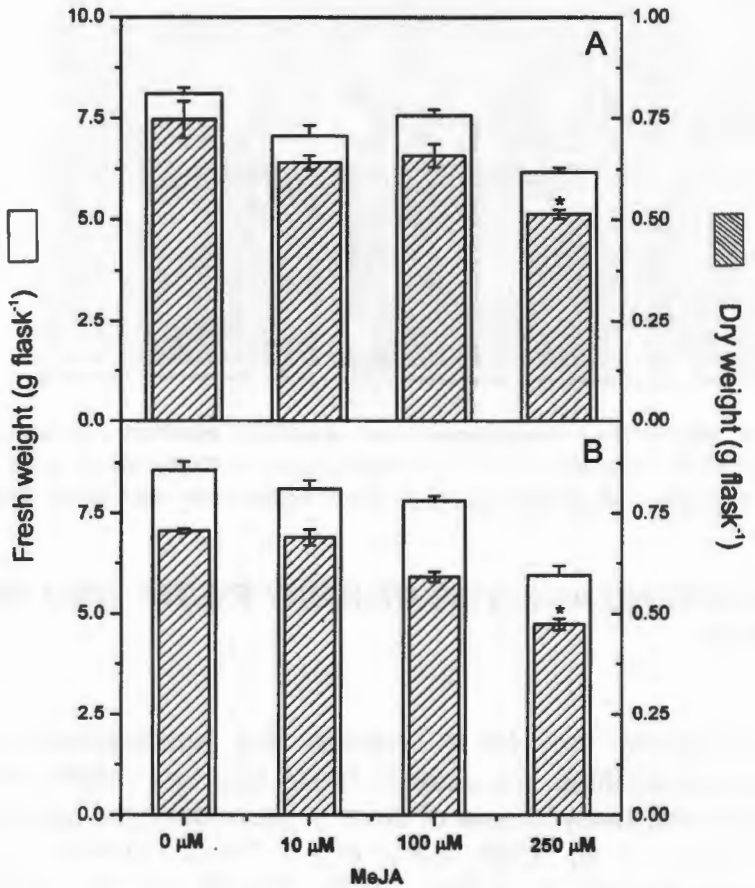

Figure 3.1. *Effect of MeJA treatment on hairy roots growth. Effect of MeJA on fresh and dry weight of hairy roots cultures after 48 h (A) and 72 h (B) of treatment. Hairy roots were treated as indicated in Materials and Methods. Error bars represent ± SE (n = 3). Asterisk represents statistical significance*

of mean differences at a given time by Turke´s test (P≤ 0.01). The experiment was made three times.

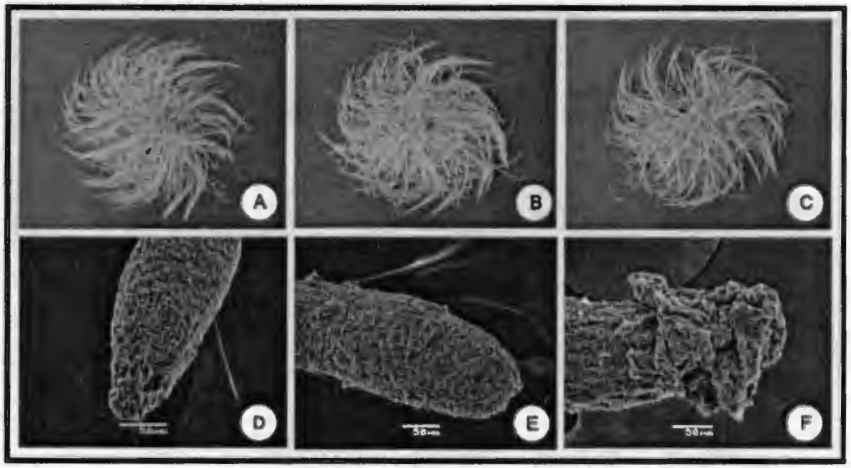

Figure 3.2. *Hairy roots phenotypes and scanning electron microscopy examination of root cap after 72 h after treatment without meja (a, d), with 10 µm (b, e) and with 100 µm MeJa (c, f). Each experiment was made three times.*

3.4.2. PROTEOME ANALYSIS OF HAIRY ROOTS TREATED WITH MEJA

It is well known that MeJA induces the accumulation of secondary metabolites. As a result, MeJA has been used as a tool to study the biosynthesis of several secondary metabolites (Vázquez-Flota *et al.*, 1994; Aerts *et al.*, 1994; Gantet *et al.*, 1998; Lee-Parsons & Ertük, 2005; Farag *et al.*, 2007; Naoumkina *et al.*, 2007; Broeckling *et al.*, 2005). It has been demonstrated that exogenous MeJA can be transported easily in the phloem (Zhang & Baldwin, 1997), and it is also found in the medium of cultured plant cells (Parchmann *et al.*, 1997).

The production of Terpene indole alkaloids (TIAs) is cellular, tissue and organ specific (De Luca & St Pierre, 2000). Significant amounts of TIAs are produced, stored and secreted by roots into the rhizosphere (Loyola-Vargas *et al.*, 2007). However, with the exception of the pathogenic related proteins (PRs), almost nothing is known about the changes in the protein pattern of the roots in response to the elicitation with MeJA.

When the hairy roots were treated with MeJA, the major change in the protein pattern was observed with 100 μM MeJA (data not shown). Therefore, the time-curse assay was done with this concentration. About 750 μg of total protein from control or treated hairy roots from 12, 24, 48 and 72 h were separated by 2-DE and visualized with Coomassie blue staining (Fig. S1).

MeJA induced global changes in the protein profiles of hairy roots (Fig. S1). Three major changes were apparent after visual analysis. First, the induction of new protein spots over the control treatment; second, the suppression of proteins that were present before MeJA treatment, and third, the quantitative variation of protein spots between time points. 108 proteins were identified representing 58 unique proteins with a MASCOT score above 60 at the $p<0.05$ level (Table 3.1). The identified proteins belong to eleven functional categories (Fig. 3.3B). The majority of the hairy root proteins were classified as carbohydrate metabolism, amino acid metabolism, cell growth and organization, protein modification and chaperones, energy and secondary metabolism; these proteins represented 69% of identified proteins in hairy roots. These proteins have been discussed in relation to jasmonates treatment in different plant culture systems (Cho *et al.*, 2007; Rakwal & Komatsu, 2000; Rakwal *et al.*, 1999), but no reports have been focused on the proteome of hairy roots so far.

Twenty different regions (panels A-T) of the 2-DE gels were selected to display the dynamic variations of proteins in these tissues (Fig. 3.3A; Figs. S2 – S8).

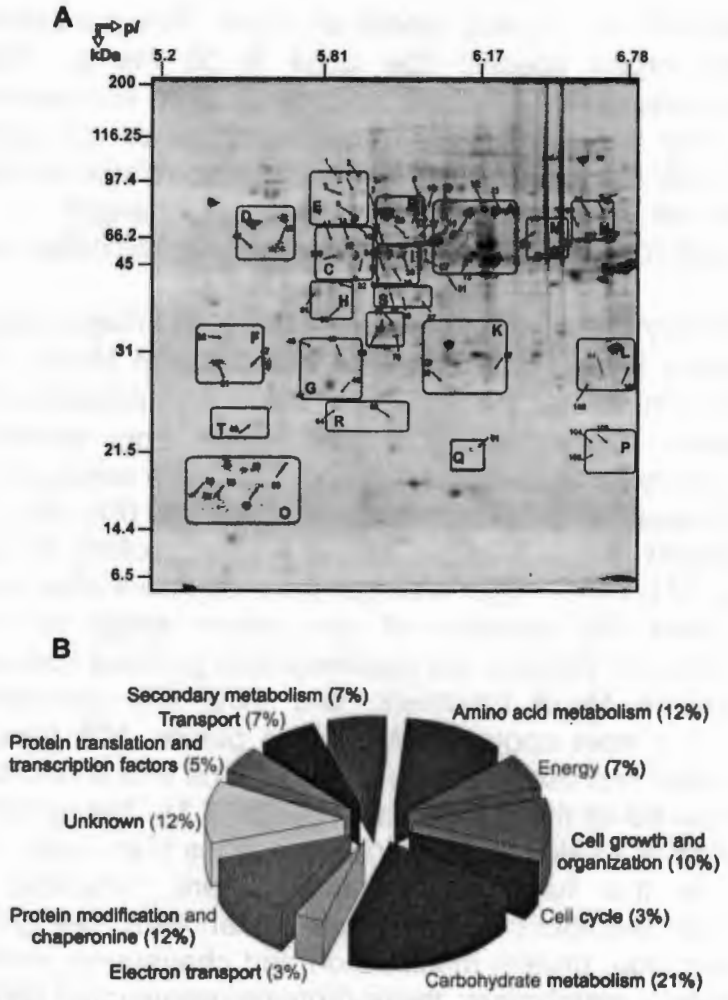

Figure 3.3. *(A) Representative 2-DE gel from 100 µM MeJA-treated hairy roots at 48 h is shown. Total protein (750 µg) was separated by 2-DE. Proteins were visualized by Coomassie Brilliant Blue R-250. The molecular masses (kDa) of protein standards (2 µl; Bio-Rad) are indicated to the left of the gel and the isoelectric point (pI) is indicated at the top of the gel. Twenty different regions (A-T) of a 2-DE gel were selected to display the dynamic variations of proteins in these tissues. The arrows with numbers represent identified proteins which are listed in Table 1. The pI and molecular masses of these protein spots varies between 5 to 6.8, and 200 to 6.5 kDa, respectively. Proteins were identified using MASCOT search engine and NCBI non-redundant protein database. Marked protein spots were analyzed by MS (Q-TOF). (B) Ontological classification of total hairy roots proteins.*

Table 3.1. List of identified proteins differentially express in *Catharanthus roseus* hairy roots after MejA exposure.

Spot No.	Protein name	PAN[1]	S[2]	P_{hum}[3]	MM	pI	Organism	Functional category
1	Xylan 1,4- -xylosidase	CAJ65921	79	2	75.80	5.19	*Populus tremula*	Cell growth and organization
2	Actin-1	P53504	151	3	41.83	5.44	*Sorghum bicolor*	Cell growth and organization
3	Glucose-6-phosphate isomerase	AAU00727	156	3	67.62	5.49	*Lycopersicon esculentum*	Carbohydrates metabolism
4	60 kDa chaperonine subunit	P06927	289	5	62.94	5.85	*Pisum sativum*	Protein modification and chaperonine
6	ATP synthase -subunit	CAA52636	140	4	59.21	5.56	*Triticum aestivum*	Energy
7	ATP synthase subunit , mitochondrial precursor	P17614	665	13	59.81	5.95	*Nicotiana plumbaginifolia*	Energy
10	ATP synthase subunit , mitochondrial	P24459	613	13	55.31	5.51	*Phaseolus vulgaris*	Energy
11	Enolase	P42896	343	5	55.31	6.51	*Ricinus communis*	Carbohydrates metabolism
12	Vacuolar H⁺-ATPase subunit B	BAF38479	297	5	54.33	5.18	*Zostera marina*	Transport
13	Vacuolar H⁺-ATPase subunit B	BAF38479	353	9	54.33	5.18	*Zostera marina*	Transport
14	Vacuolar ATP synthase catalytic subunit A	Q40002	67	2	64.05	5.38	*Hordeum vulgare*	Transport
15	*S*-adenosylmethionine synthetase 1	Q96551	291	7	43.02	5.59	*Catharanthus roseus*	Amino acid metabolism

Table 3.I. Continued

17	Actin-1	P30164	102	2	41.69	5.4	*Pisum sativum*	Cell growth and organization
18	Actin-1	P30164	164	4	41.69	5.4	*Pisum sativum*	Cell growth and organization
21	1-deoxy-D-xylulose-5-phosphate reductoisomerase	AAF65154	333	7	51.13	6.1	*Catharanthus roseus*	Secondary metabolism
24	Peroxidase 1	CAJ84723	190	4	33.90	7.63	*Catharanthus roseus*	Secondary metabolism
26	*S*-adenosylmethionine synthetase 2	Q96552	356	8	42.97	5.51	*Catharanthus roseus*	Amino acid metabolism
29	Hypothetical protein	T14439	91	2	28.89	4.94	*Brassica oleracea*	Unknown
31	*O*-methyltransferase	AAM97498	533	10	38.76	5.58	*Catharanthus roseus*	Secondary metabolism
32	*S*-adenosylmethionine synthetase 2	Q96552	167	3	42.97	5.51	*Catharanthus roseus*	Amino acid metabolism
33	20S proteasome 6 subunit	AAN07899	260	4	29.85	5.07	*Nicotiana benthamiana*	Protein modification and chaperonine
34	*S*-adenosylmethionine synthetase 2	Q96552	167	3	42.97	5.51	*Catharanthus roseus*	Amino acid metabolism
35	Unknown	AAX19869	60	2	33.60	4.93	*Doryanthes excelsa*	Unknown
36	Glyoxalase/bleomycin resistance protein/dioxygenase	ABE89103	93	2	32.22	5.13	*Medicago truncatula*	Unknown
37	Cysteine synthase	AAR18402	205	3	34.01	5.71	*Nicotiana plumbaginifolia*	Amino acid metabolism

Table 3.I. Continued

41	Heat shock protein 70	AAM48131	113	4	70.75	5.14	*Saussurea medusa*	Protein modification and chaperone
42	Heat shock cognate 70 kDa protein 2	P27322	206	6	70.66	5.08	*Solanum lycopersicum*	Protein modification and chaperone
46	Triosephosphate isomerase, chloroplast precursor	P48496	72	2	34.38	6.45	*Spinacia oleracea*	Carbohydrates metabolism
49	Putative 3 proteasome subunit	CAC43324	66	1	19.83	5.08	*Nicotiana tabacum*	Protein modification and chaperone
60	Adenosine kinase isoform 2S	AAU14833	112	3	37.53	5.16	*Nicotiana tabacum*	Cell cycle
61	Eukaryotic translation initiation factor 5A-2	Q9AXQ5	82	2	17.57	5.78	*Solanum lycopersicum*	Protein translation and transcription factor
63	Benzoquinone reductase	ABN12321	212	2	21.65	6.09	*Gossypium hirsutum*	Electron transport
64	Putative protein	CAA19721	90	1	22.29	6.3	*Arabidopsis thaliana*	Unknown
65	Triose -phosphate isomerase	CAI43251	86	2	27.18	5.87	*Phaseolus vulgaris*	Carbohydrates metabolism
67	Alcohol dehydrogenase	CAJ21172	249	4	40.97	6.28	*Alnus glutinosa*	Carbohydrates metabolism
68	Isocitrate dehydrogenase	CAD24779	258	6	46.14	6.0	*Cucumis sativus*	Carbohydrates metabolism
71	*S*-adenosylmethionine synthetase 3	P50303	239	6	39.48	6.2	*Actinidia chinensis*	Amino acid metabolism
75	Actin	AAB07498	73	2	31.43	5.13	*Chlorella vulgaris*	Cell growth and organization
81	Alpha chain of nascent polypeptide associated complex	BAF46352	104	3	21.91	4.32	*Nicotiana benthamiana*	Protein translation and transcription factor

Table 3.I. Continued

83	Proteasome subunit alpha type-5	Q9M4T8	93	2	25.96	4.7	*Glycine max*	Protein modification and chaperone
84	Translationally-controlled tumor protein homolog	Q944T2	89	2	18.98	4.57	*Glycine max*	Cell cycle
85	Lipid-associated family protein	NP_195683	64	1	20.12	4.97	*Arabidopsis thaliana*	Unknown
87	Alcohol dehydrogenase	CAJ21172	161	2	40.97	6.28	*Alnus glutinosa*	Carbohydrates metabolism
89	Alcohol dehydrogenase	CAJ21172	161	2	40.97	6.28	*Alnus glutinosa*	Carbohydrates metabolism
90	Pyruvate dehydrogenase E1 component subunit beta, mitocondrial precursor	P52904	251	4	38.76	5.88	*Pisum sativum*	Carbohydrates metabolism
91	Fructokinase	AAA80675	268	5	35.42	5.38	*Beta vulgaris*	Carbohydrates metabolism
92	Putative cysteine proteinase RD21A precursor	BAC43113	68	1	40.27	4.77	*Arabidopsis thaliana*	Protein modification and chaperone
93	14-3-3-like protein	P29307	149	4	29.23	4.61	*Oenothera elata*	Protein translation and transcription factor
95	F1-ATPase alpha subunit mitochondrial	AAB03873	91	2	55.17	5.84	*Petunia axillaris*	Transport
98	*S*-adenosylmethionine synthetase 3	P43282	79	3	42.62	5.76	*Solanum lycopersicum*	Amino acid metabolism
99	RGP1 protein	CAA09469	111	3	39.45	8.21	*Oryza sativa*	Cell growth and organization
102	Alcohol dehydrogenase 2	ABC56182	87	2	22.73	5.29	*Paeonia anomala*	Carbohydrates metabolism

Table 3.I. Continued

104	Nucleoside diphosphate kinase	AAF08537	62	1	25.24	9.37	*Pisum sativum*	Energy
105	Ubiquinol-cytochrome-c reductase subunit II	P29677	129	2	54.64	5.71	*Solanum tuberosum*	Electron transport
106	Os08g0536000	NP_001062360	188	3	39.91	5.25	*Oryza sativa*	Unknown
107	NADPH-specific isocitrate dehydrogenase	AAA33978	61	2	49.10	6.13	*Glycine max*	Carbohydrates metabolism
108	Os02g0653300	NP_001047604	64	1	15.58	10.61	*Oryza sativa*	Unknown

¶: protein accession number (NCBI); †: Mascot MOWSE score; ‡: number of peptides matched to the protein; MW: molecular weight; pI: isoelectric point.

3.4.2.1 CARBOHYDRATE METABOLISM

Several proteins of this category decreased in the accumulation pattern during the time-curse assay. Among these proteins, the enolase (spot 11; Table 3.1; Fig. S2A; EC 4.2.1.11) and the fructokinase (Table 3.1; spot 91; Fig. S4H; EC 2.7.1.4) were identified. Enolase catalyzes the glycolytic reaction where a molecule of water is reversibly removed from 2-phosphoglycerate to phosphoenolpyruvate. Fructokinase is involved in primary metabolism in sink tissues (Kanayama *et al.*, 1998). Three different isomerases were also identified (spots 3, 46 and 65; Table 3.1). Spot 3 (Table 3.1; Fig. S3E) was identified as glucose-6-phosphate isomerase (EC 5.3.1.9) and spots 46 and 65 (Fig. S4G - S6O) were identified as triosephosphate isomerases (EC 5.3.1.1). A considerable decrease of spot 90 (Fig. S2C), identified as pyruvate dehydrogenase E1 component subunit β (E1; EC 1.2.4.1; Table 3.1) was observed throughout the induction time. The spot 60 (Fig. S7R) corresponding to an isocitrate dehydrogenase (Table 3.1; EC 1.1.1.42) decreased after 24 and 48 h of treatment, but spot 107 (Fig. S6N) identified as NADPH-isocitrate dehydrogenase (Table 3.1; EC 1.1.1.42) transiently increased during the induction time. It has been suggested that this NADPH-isocitrate dehydrogenase is involved in the supply of 2-oxoglutarate for ammonia assimilation and glutamate synthesis in higher plants through the glutamine synthetase/glutamate synthase (GS/GOGAT) cycle (Miflin & Lea, 1982). Four differential spots (67, 87, 89 and 102; Figs. S6M, S5K, S5K, and S5L) were identifies as alcohol dehydrogenase (ADH; EC 1.1.1.1; Table 3.1). Spots 87 and 89 increased at first half of induction, but the spots 67 and 102 decreased after 48 h of elicitation with MeJA. ADH is associated with different stress responses in plants, such as anoxia (Ricard *et al.*, 1986) and fruit ripening(Prestage *et al.*, 1999).

3.4.2.2. AMINO ACID METABOLISM

Six spots (15, 26, 32, 34, 71 and 98; Figs. S2A, S4I, S4H, S2C, S2A and S6N) identified as *S*-adenosylmethionine synthetase (SAMS; EC 2.5.1.6; Table 3.1) increased during different times of treatment when compared to the control. The spots 26, 32 and 34 were identical to SAMS 2, the spot 15 to SAMS 1 and the spots 71 and 98 to SAMS 3. Differential expression of SAMS has been observed under a variety of stresses (elicitor, nutritional down-shift, increased NaCl and MeJA) (Schröder *et al.*, 1997; Imanishi *et al.*, 1998). Overexpression or silencing of SAMS in tobacco resulted in yellow-green leaves and stunted phenotypes, respectively (Boerjan *et al.*, 1994), and anti-sense expression of SAMS led to stunting and increased cytokinin levels (Masuta et al., 1995). In addition, spot 37 (Fig. S8S) identified as cysteine synthase (EC 4.2.99.8; Table 3.1) increased during the last phase of induction. This enzyme catalyses the formation of cystein from *O*-acetylserine and bisulphide that is responsible for the incorporation of inorganic sulphur into the amino acid cysteine, which can be subsequently converted into other sulphur-containing compounds, such as methionine or incorporated into the tripeptide glutathione (Warrilow & Hawkesford, 1998).

3.4.2.3. PROTEIN MODIFICATION AND CHAPERONES

The proteasome is a multicatalytic proteinase complex which is characterized by its ability to catabolize Ub-protein conjugates (Smalle & Vierstra, 2004). The 20S proteasome of eukaryotes consists of two copies, each one of 7 distinctive α- and 7 distinctive β-type subunits (Voges *et al.*, 1999). In hairy root elicited with MeJA, a slightl change in the spots 33, 49 and 83 (Figs. S4H, S4G and S8T) identified as the proteasome subunits (Table 3.1), were observed. Spots 33 and 49 identified as 20S proteasome alpha 6 subunit (EC 3.4.25.1; Table 3.1)

decreased in the hairy root treated with MeJA throughout the time-course assay. On the other hand, the spot 83 identified as 20S proteasome subunit α type-5 (EC 3.4.25.1; Table 3.1) increased at 24 h. From there, the spot 83 followed the same pattern at the last time point of induction (72 h). Significant increases of transcript α 6 subunit were observed after external stimuli, including MeJA, NaCl and salicylic acid (Kim *et al.*, 2003). However, the ubiquitin/26S proteasome pathway is one of the most elaborated regulatory mechanisms in *A. thaliana*, being composed for more than 1300 genes and representing almost 5% of the whole proteome (Smalle & Vierstra, 2004). In addition, spots 41 and 42 (Fig. S3D) were identified as heat shock protein 70 (HSP70) and heat shock cognate 70 kDa protein 2 (HSC70s), respectively (Table 3.1). Both spots increased after 48 h of the treatment with MeJA. Most HSP70s are expressed under environmental stress, but HSC70s are also expressed under normal conditions. These proteins are often assist in the folding of *de novo* synthesized polypeptides and the import/translocation of precursor proteins (Sung *et al.*, 2001; Frydman, 2001). On the other hand, spot 92 (Fig. S3F) identified as putative cysteine proteinase RD21A precursor (EC 3.4.22; Table 3.1) increased in the hairy roots treated with MeJA at 24 and 72 h. Cysteine proteinase (EC 3.4.22) expressed in roots was suggested to play a role in the protection of root tissue against insects invading the root or the developing root of seedling (Lim *et al.*, 1996). Furthermore, a cysteine proteinase was induced by wounding and JA treatment (Pernas *et al.*, 2000). Therefore, it is possible to suggest a correlation of these proteins with the metabolic status of hairy roots.

3.4.2.4. CELL GROWTH AND ORGANIZATION

The decreased spot 99 (Fig. S6N) was identified as reversibly glycosylated polypeptides (RGPs; EC 2.4.1.112; Table 3.1). In dicotyledonous plants, 20% of the primary cell wall consists of the polysaccharide xyloglucan, whereas in monocotyledonous

this hemicellulose makes up 2% of the primary cell wall (Darvill *et al.*, 1985). RGP1 has been implicated in xyloglucan biosynthesis and possible other hemicelluloses (Dhugga *et al.*, 1997). However, the regulatory mechanism of hemicellulose structure is difficult to establish because the number of enzymes characterized so far is limited. In addition, spot 1 (Fig. S3E) identified as xylan 1, 4-β-xylosidase (Table 3.1) was present only in the control at 24 and 48 h. These enzymes act cooperatively to convert xylan into its constituting sugars (Rahman *et al.*, 2003). Furthermore, four spots (2, 17, 18 and 75; Figs. S3E, S2C, S2C and S4I; Table 3.1) were identified as actin protein. The spots 2, 17 and 18, which are identical to the actin-1, decreased after 48 h in the treatment. This protein is an important determinant in cytoplasmic streaming, cell shape determination, cell division, organelle movement, and extension growth (McElroy *et al.*, 1990). MeJA appears to promote the rapid decrease in the rates on transcription of the actin genes in barley leaves (Reinbothe *et al.*, 1997). However, the spot 75 identified also as actin increased from 24 to 72 h after MeJA treatment.

3.4.2.5. ENERGY

Three protein spots (6, 7 and 10; Figs. S3E, S2B and S2A; Table 3.1) were identified as ATP synthase. These proteins were differentially expressed in hairy roots of *C. roseus* treated with MeJA. Spots 6 and 7 that match to the identity of ATP synthase β-subunit mitochondrial precursor (EC 3.6.1.34) decreased after 24 and 48 h of treatment. However, spot 10 identified as ATP synthase α subunit mitochondrial precursor (EC 3.6.1.14) increased during the first 24 h and then decreased after 48 h in the hairy roots treated. In addition, the spot 104 (Fig. S7P) that decreased at 24 h of induction was identified as nucleoside diphosphate kinase (NDPK; EC 2.7.4.6; Table 1). NDPK catalyses the transference of the c-phosphate of a donor nucleoside triphosphate (NTP) to an acceptor nucleoside diphosphate (NDP). While ATP is generally

considered as the preferred donor of phosphate *in vivo*, NDPK also transfers the c-phosphate from other NTPs donors (Lascu & Gonin, 2000). This enzyme is widely distributed and it is believed to play a role in the general homeostasis of the cellular nucleoside triphosphate pools. In prokaryotes, NDPK has been shown to perform a metabolic housekeeping function because of its involvement in the generation of NTPs from ATP (Bernard *et al.*, 2000). Furthermore, NDPK function is associated with heat stress (Galvis *et al.*, 2001) and growth (Yano *et al.*, 1995). Moreover, constitutive over-expression of NDPK2 in Arabidopsis (*At*NDPK2) confers an enhancing tolerance to multiple environmental stresses (Moon *et al.*, 2003). This result suggests that the capability of ATP synthesis by oxidation of biological fuels decrease in the hairy root of *C. roseus*.

3.4.2.6. SECONDARY METABOLISM

In hairy roots two spots (20 and 21; Fig. S2B) differentially expressed were identifie as 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR; EC 1.1.1.267; Table 3.1). Spot 20 is present exclusively in the hairy roots treated with MeJA at 24 h and transiently increased until 72 h after the treatment. A similar expression pattern was observed for spot 21 (DXR; EC 1.1.1.267; Table 3.1), which increased from 24 h to the end of treatment. In higher plants, the isoprenoid building unit is formed by two pathways that operate in different subcellular compartments (Eisenreich *et al.*, 2001; Rohmer *et al.*, 1993; Lichtenthaler *et al.*, 2000). The well-known mevalonate (MVA) pathway in the cytosol and the 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway in the plastids. In MEP pathway the conversion of 1-Deoxy-D-xylulose 5 phosphate (DXP) to 2-*C*-methyl-D-erythritol 4-phosphate (MEP) is catalyzed by the DXR. Peroxidase 1 was also differentially accumulated (spot 24; EC 1.11.1.7) in the hairy roots treated with MeJA. This protein increased at 12 and 72 h after induction, but at 24 and 48 h only was present in the control. Moreover, spot 31 identified as flavonoid *O*-methyltransferase (OMT; EC 2.1.1.6) increased at

24 and 72 h of treatment. OMT performs two sequential methylations at the 3' and 5' positions of the B-ring in myricetin (flavonol) and dihydromyricetin (dihydroflavonol) in cell suspension cultures of *C. roseus* (Cacace *et al.*, 2003).

3.4.2.7. CELL CYCLE

Spot 84 (Fig. S4G) was identified as a translationally-controlled tumor protein homolog (TCTP) and it increased after 24 and 48 h of treatment with MeJA. Biochemical and immunofluorescence studies demonstrated that TCTP is a tubulin-binding protein that associates with microtubules in a cell-cycle dependent fashion (Gachet *et al.*, 1999). Recently, the polo-like kinase was shown to directly interact with a phosphorylate TCTP and was shown to be required for the normal progression of cytokinesis (Yarm, 2002). Moreover, a decrease of spot 60, identified as adenosine kinase isoform 2S (ADK; EC 2.7.1.20; Table 3.1), was observed. This protein is a modulator of root cap morphogenesis and gravitropism (Young *et al.*, 2006).

The other remaining functional categories correspond to a minor number of proteins that were identified in this study. Spots 12 and 13 (Figs. S2A and S2B) were identified as vacuolar H^+-ATPase subunit β and spot 14 was identified as the subunit α, both increased differentially with the MeJA treatment. Spot 96, identified as F_1-ATPase α subunit also increased at 24 and 48 h after exposure with MeJA. Translation initiation factor 5A-2 (eIF-5A, spot 61; Fig. S7Q; Table 3.1) and 14-3-3-like protein (spot 93; Fig. S5J) also showed an increased expression pattern after 48 h of treatment. Both proteins are involved in the regulation of cell proliferation, cell growth, programmed cell death and several stress response (Li *et al.*, 2004; Thompson *et al.*, 2004; Kidou *et al.*, 1993). 14-3-3-like proteins were also induced in response to infection by the powdery mildew fungus *Erysiphe graminis* (Brandt *et al.*, 1992). On the other hand, after 24 h of treatment, spot 81, the α-chain of nascent polypeptide associated complex (NAC), decreased

significantly. This protein is a heterodimeric complex composed of an α and β-subunit (Rospert *et al.*, 2002). It has been shown that these proteins interact with the L25 ribosomal protein near the site where newly synthesized polypeptide chains emerge (Grallath *et al.*, 2006). In addition, the decrease of a benzoquinone reductase (spot 63; EC 1.6.5.7; Fig. S6O) and ubiquinol-cytochrome-C reductase subunit II (spot 105; EC 1.10.2.2; Fig. S3E) was also observed.

3.5. DISCUSSION

Growth regulators are not only instrumental in regulating developmental processes in plants, but also play an important role in plant's response to biotic and abiotic stresses (Creelman & Mullet, 1997; Aerts *et al.*, 1994; Gantet *et al.*, 1998; Rijhwani & Shanks, 1998). In some extend, research has focused on MeJA effect because of the faculty to disturb the secondary metabolism of several plant cultures (Godoy-Hernández & Loyola-Vargas, 1997; Vázquez-Flota & De Luca, 1998; Vázquez-Flota *et al.*, 1994; Martin *et al.*, 2002; Elisabetsky *et al.*, 1997). Considering the importance as well as the lack of detailed knowledge about the role of MeJA on plants under abnormal physiological condition, which includes stress and disease, it would be useful to have detailed insights into several plant systems. The systematic study provides new and detailed information about the effect of MeJA on both the morphological responses and molecular changes at the protein level. Therefore, in the present study, we treated hairy roots of *C. roseus* with MeJA and used a proteomics approach to detect and identify the MeJA-responsive proteins in roots. With the results shown in this work, we were able to reconstruct a proteomics-based metabolic pathway for the treated hairy root cultures with MeJA (Fig. 3.4) which provided a better understanding of the hairy roots response to MeJA.

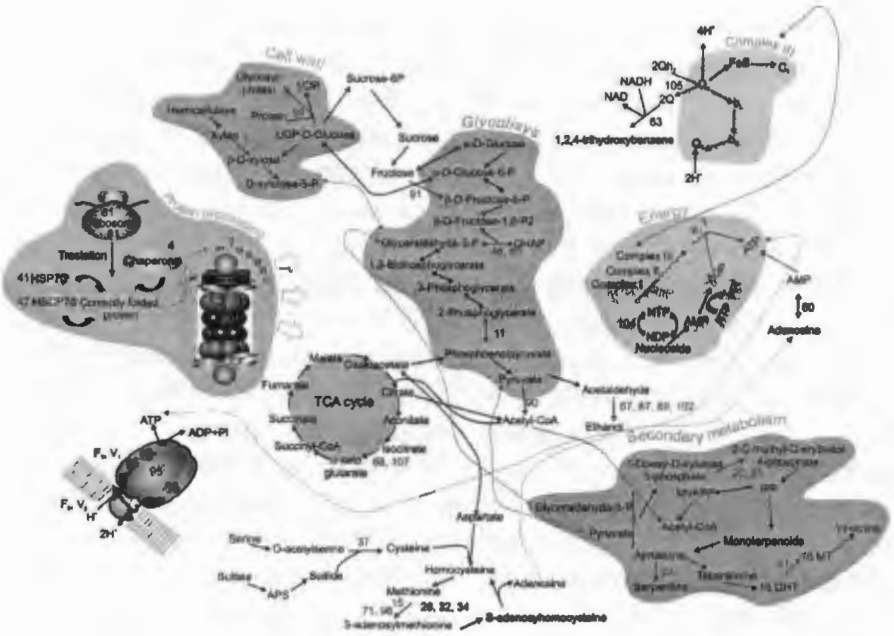

Figure 3.4. *Proteomics-based metabolic pathway reconstruction for the treated hairy root cultures with MeJA. Red arrows and numbers correspond to biochemical functions identified from the proteome of hairy roots (Table 3.1). Colored blocks represent known metabolic modules (Buchanan, Gruissem, and Jones 2000). Black arrows represent individual functions undetected in the metabolic modules. Dotted black lines represent several consecutive functions undetected in these modules. Gray arrows materialize transfers of metabolites between different modules.*

The negative effect of MeJA on root growth has been described in the model plant *A. thaliana* (Staswick *et al.*, 1992). Similar observations were made in cell suspension cultures of tobacco (Goossens *et al.*, 2003) and recently, in rice root (Cho *et al.*, 2007). Several proteins belonging to carbohydrate metabolism decreased considerably (Fig. 3.4; spots 11, 91, 46, 65, 60 and 90). Furthermore, actin proteins (Fig. 3.4; spots 2, 17, 18 and 75) which are determinant in cytoplasmic streaming, cell shape determination, cell division, organelle movement, and extension

growth (McElroy *et al.*, 1990) also decreased. On the other hand, the increase of HSC70 (Fig. 3.4; spot 42) has been correlated with negative effect of the plants' size and root system (Sung & Guy, 2003). In the same way, the induction of ADHs (Fig. 3.4; spots 87 and 89), which are associated with anoxia, were also observed (Ricard *et al.*, 1986). Changes in the ADH activity alters the production of C6-volatiles (Bate *et al.*, 1998), which have the potential to control pest insects and pathogens (Vancanneyt *et al.*, 2001; Zhang *et al.*, 1999). Interesting, there is a market correlation with the release of C6-volatiles and the increase of JA biosynthesis (Engelberth *et al.*, 2004). JA and C6-volatiles share the same important intermediate, the 13-hydroperoxy-linolenic acid (the product of action of lipoxygenase on linolenic acid) (Gatehouse, 2002). These results show the decrease of proteins involved in the metabolism of carbohydrate. In the same scenario, the differential induction of an alternative anaerobic pathway is observed, which might account for hairy root growth differences between the treatment with MeJA and respective control. Deficient ATP production in mitochondria could playing a crucial role in the decrease of growth rate by MeJA treatment (Cho *et al.*, 2007). ROS production increased in the mitochondria at the first hour of treatment with MeJA which caused a series of alterations in mitochondrial dynamics including the cessation of mitochondrial movement, the loss of mitochondrial transmembrane potential (MPT), and the morphological transition and aberrant distribution of mitochondria followed with a subsequent cell death (Zhang & Xing, 2008).

Proteins (Fig. 3.4; spot 84: TCTP and spot 60: ADK) involved in cell cycle also decreased in the hairy root elicited with MeJA. Mutation in one of two Arabidopsis ADK genes, ADK1, results in cap morphogenesis defects, along with alterations in root sensitivity to gravistimulation and slower kinetics of root gravitropic curvature (Young *et al.*, 2006). Similar, in the present study, the decreased ADK is correlated with cap morphogenesis anomaly (Figs. 2E and 2F) in our hairy roots cultures. The detrimental effect on hairy root growth is

correlated with the overproduction of several compounds such as TIAs (Ruíz-May *et al.*, 2008).

In the present work, DXR (Fig. 3.4; spots 20 and 21; Table 3.1) showed an increased expression pattern in the hairy roots treated with MeJA. DXP is a precursor not only for isoprenoids but also for the cofactors thiamine pyrophosphate and pyridoxal phosphate (Julliard & Douce, 1991; Julliard, 1992). The reaction catalyzed by DXR is actually the first committed step of the MEP pathway. Therefore, DXR could play an important role in the control of plastid isoprenoid biosynthesis. In agreement with our results, positive correlation was found between the accumulation of *DXR* transcript and terpenoid indole alkaloids in periwinkle cell suspension culture (Veau *et al.*, 2000). Vacuolar peroxidase (Fig. 3.4; spot 24; Table 3.1) was also differentially expressed in the hairy root elicited with MeJA. *In vitro* studies have shown the capacity of plant peroxidases to accept as substrates a number of vacuolar metabolites such as phenols, flavonoids and alkaloids. It has been suggested that, *in vivo*, peroxidases assume specific functions in the metabolism of these compounds (Sottomayor *et al.*, 1996; Sottomayor & Barceló, 2003; Sottomayor *et al.*, 2004).

Taking together these results, it is possible to infer that the hairy roots cultures change their metabolic status when they are treated with MeJA. There is a decrease in the basic metabolism which correlates with the inhibition of growth and the effect in the morphology of the root cap. On the other hand, proteins involved in secondary metabolism showed an increased expression pattern throughout the induction with the possible overproduction of C6-volatiles which is directly correlated with the jasmonates effects.

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3.7. Figures supplementary

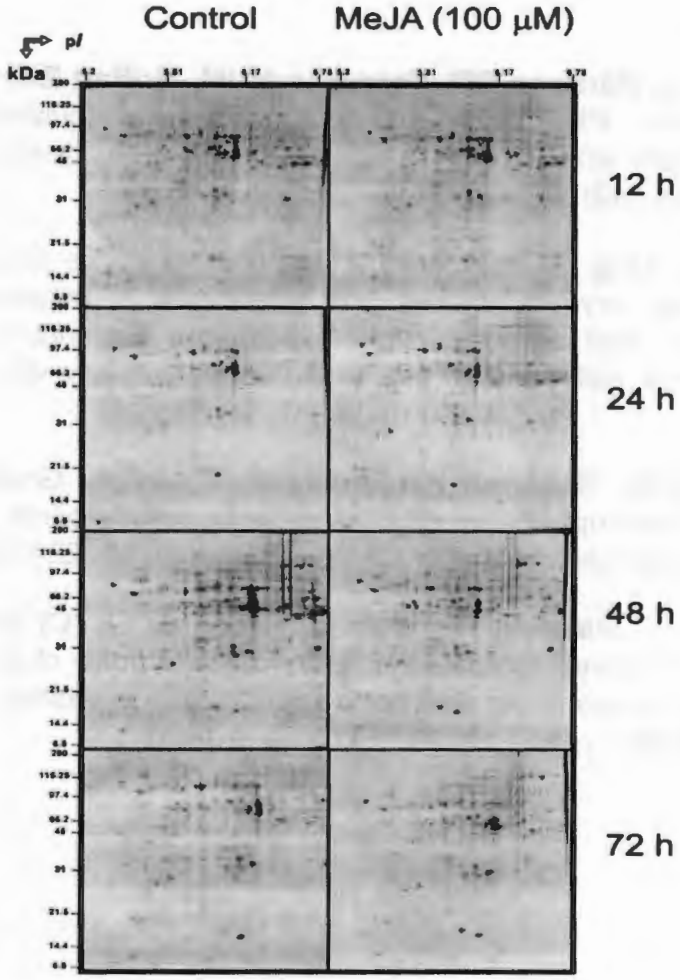
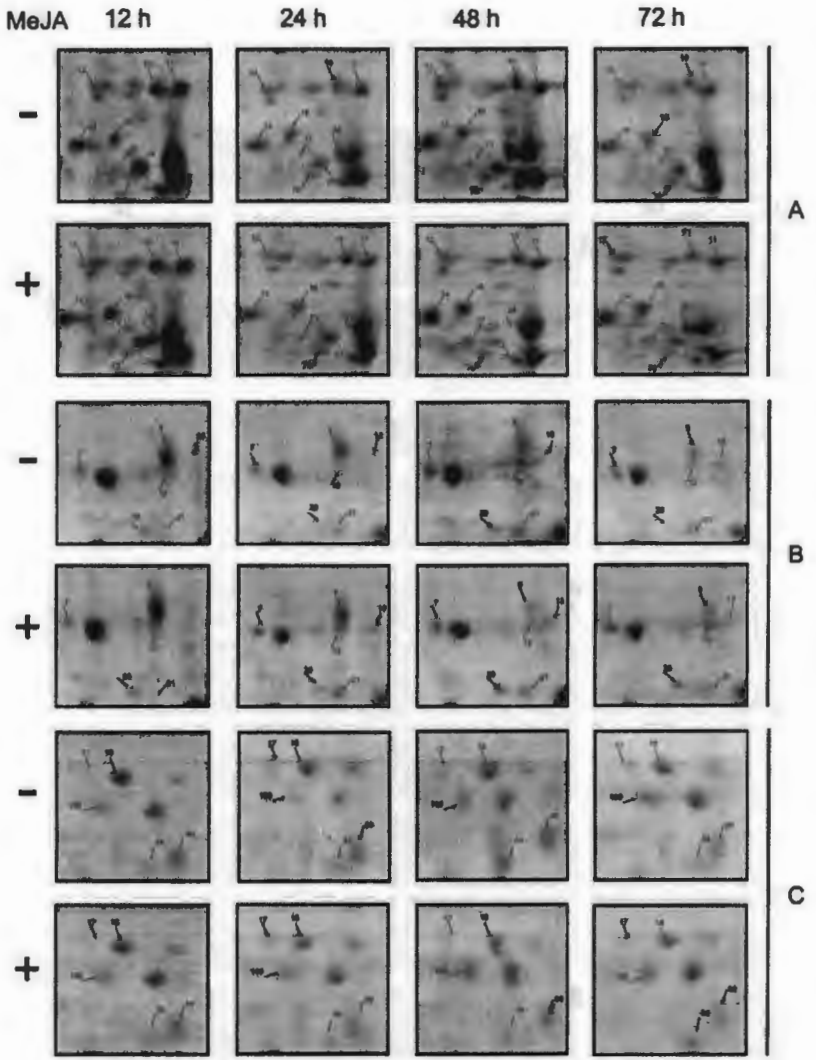


Figure S1. *Two-dimensional electrophoresis of total hairy root proteins after MeJA treatment. A representative proteomic map shows root-tissue proteins at 12, 24, 48 and 72 h after treatment of C. roseus hairy roots tissues alone (control) or treated with 100 µM MeJA. Seven hundred and fifty micrograms of protein were focused and separated as described under "Materials and Methods." The molecular masses (kDa) of protein standards are indicated to the left of the gel, and the isoelectric point (pI) is indicated at the top of the gel. Protein spots were visualized by staining with Coomassie Brilliant Blue R-250.*



Figures S2. *Dynamic variations of root protein profiles by 2-DE. Three different regions (boxed in Figure 3.3A as A, B and C) of a 2-DE gel from hairy roots were selected to display the dynamic variations of proteins in these tissues. The technical conditions are described on the legend of Figure S1 and under Materials and Methods.*

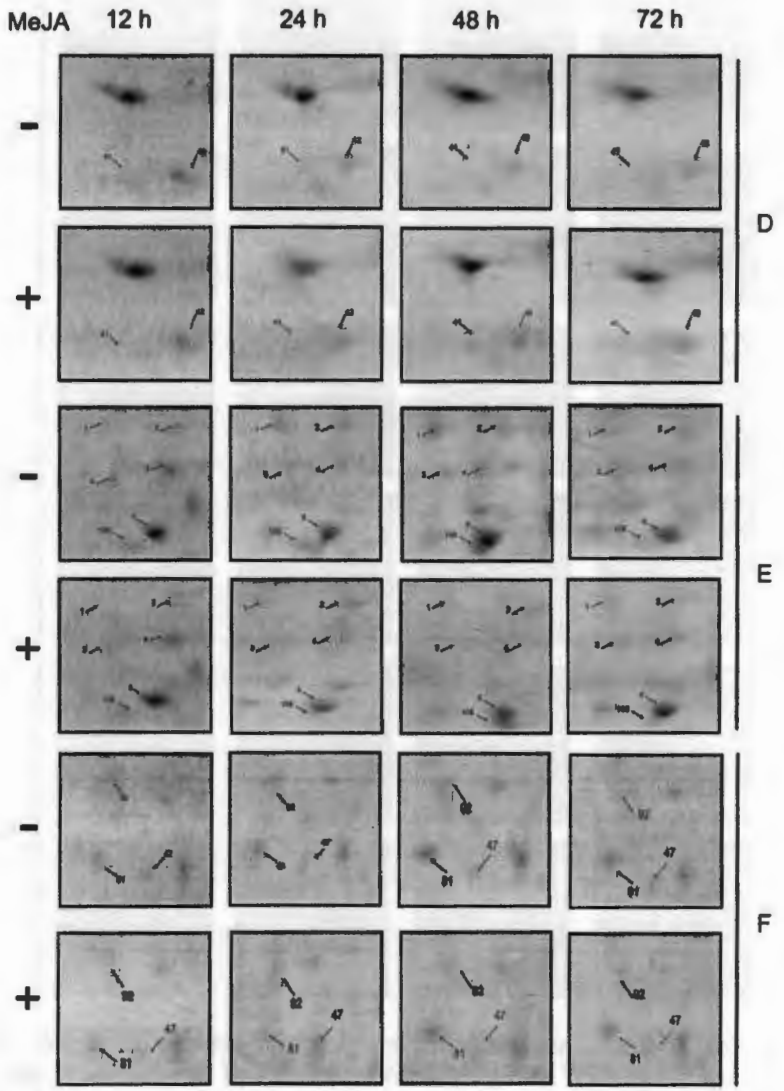


Figure S3. *Dynamic variations of root protein profiles by 2-DE. Three different regions (boxed in Figure 3.3A as D, E and F). Total protein (750 μg) was separated by 2-DE in each time of treatment. Protein spots were visualized by staining with Coomassie Brilliant Blue R-250.*

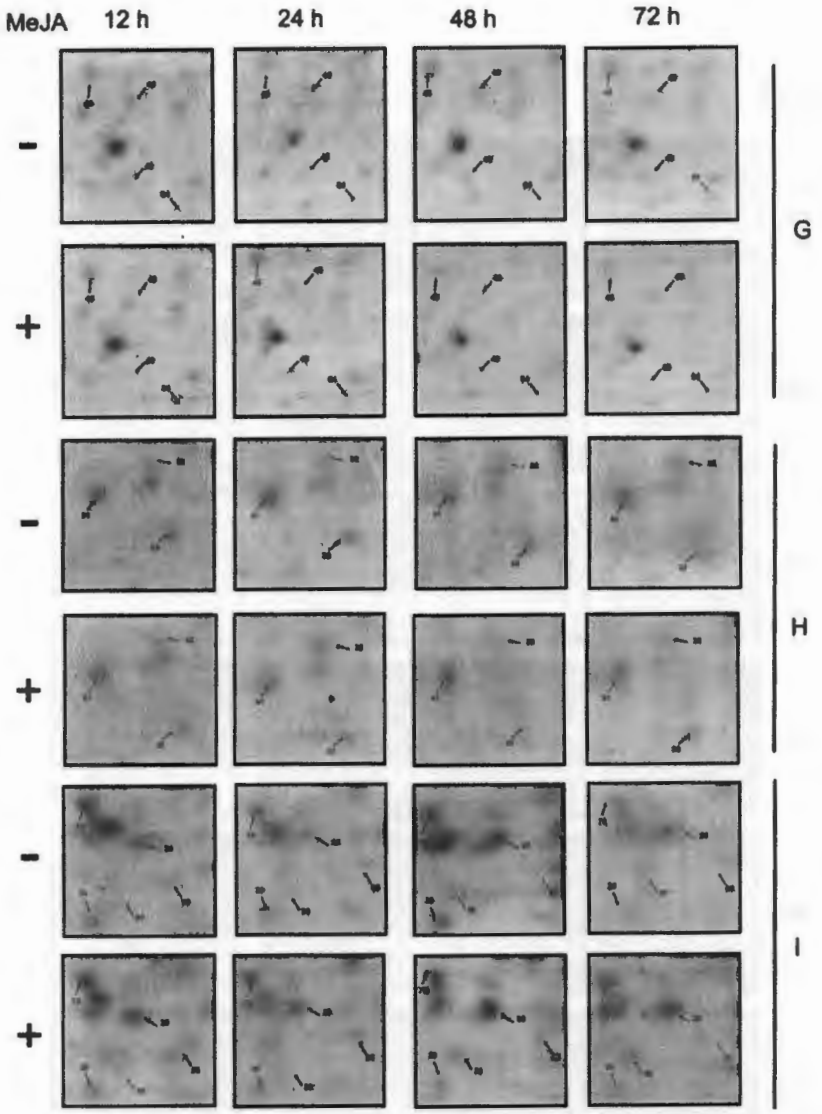


Figure S4. *Dynamic variations of root protein profiles by 2-DE. Three different regions (boxed in Figure 3.3A as G, H and I). Total protein (750 μg) was separated by 2-DE in each time of treatment. Protein spots were visualized by staining with Coomassie Brilliant Blue R-250.*

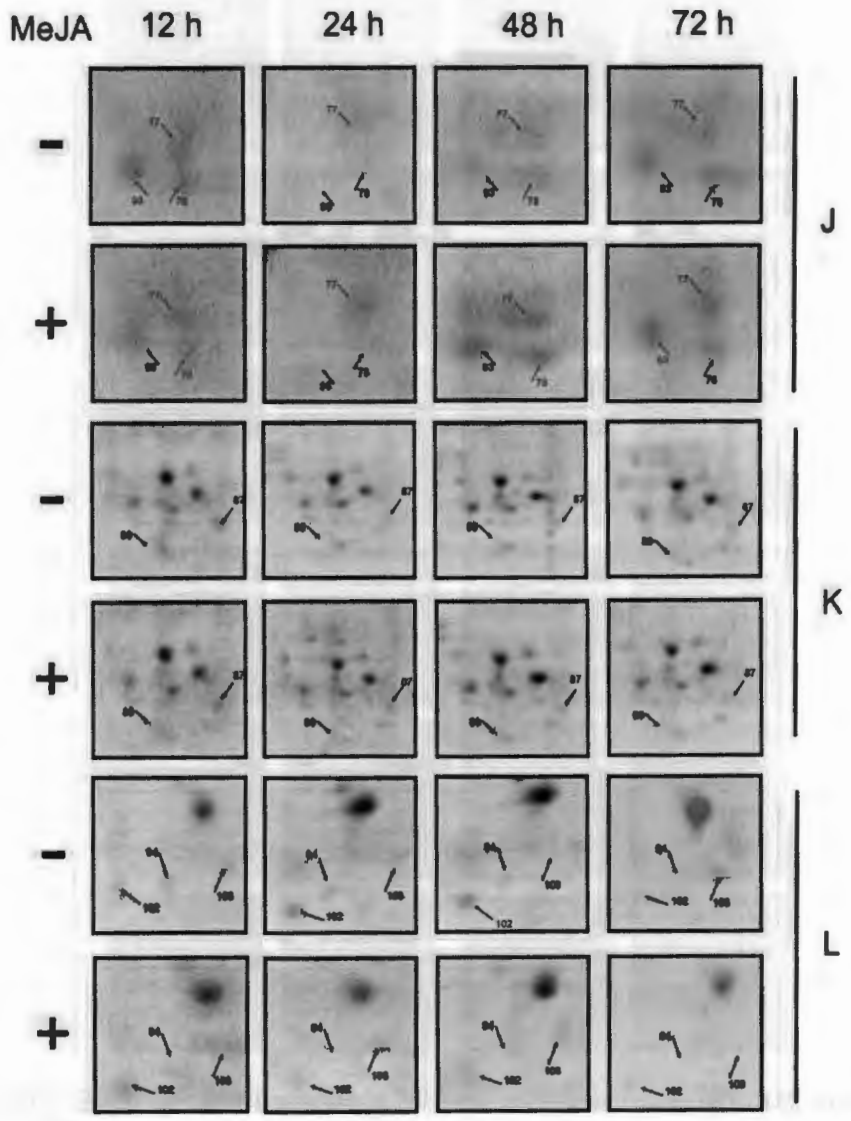


Figure S5. *Dynamic variations of root protein profiles by 2-DE. Three different regions (boxed in Figure 3.3A as J, K and L). Total protein (750 μg) was separated by 2-DE in each time of treatment. Protein spots were visualized by staining with Coomassie Brilliant Blue R-250.*

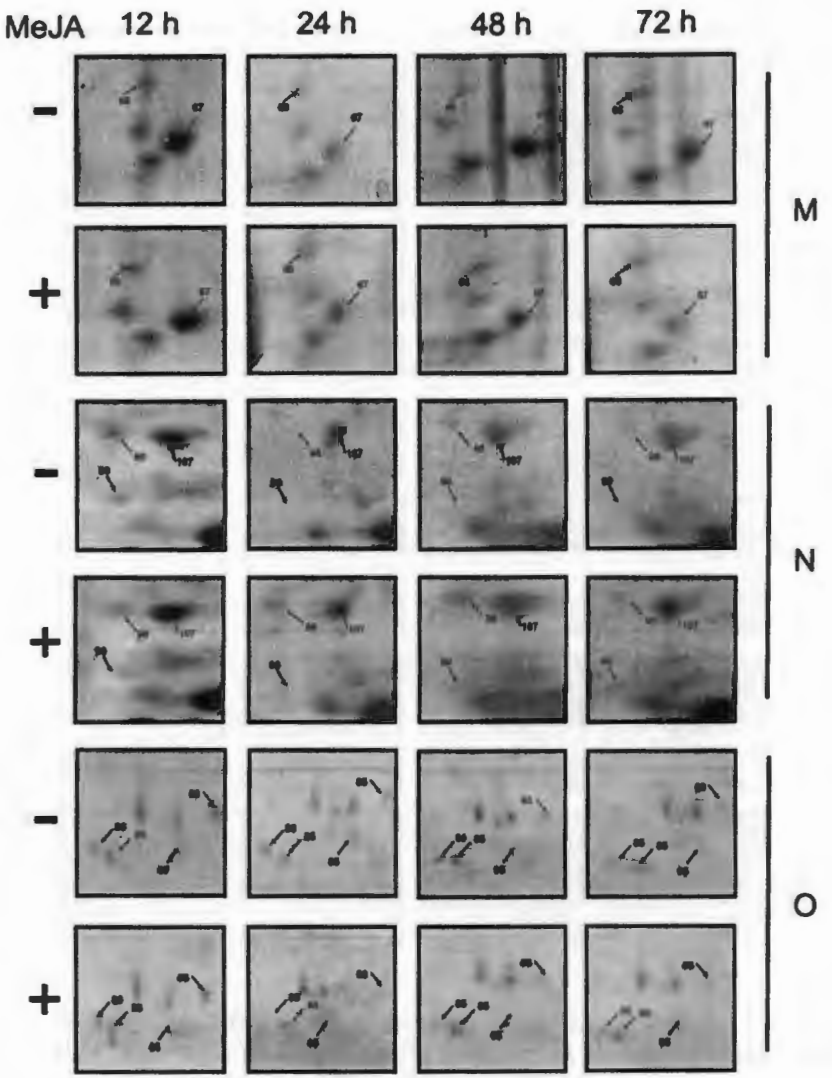


Figure S6. *Dynamic variations of root protein profiles by 2-DE. Three different regions (boxed in Figure 3.3A as M, N and O). Total protein (750 µg) was separated by 2-DE in each time of treatment. Protein spots were visualized by staining with Coomassie Brilliant Blue R-250.*

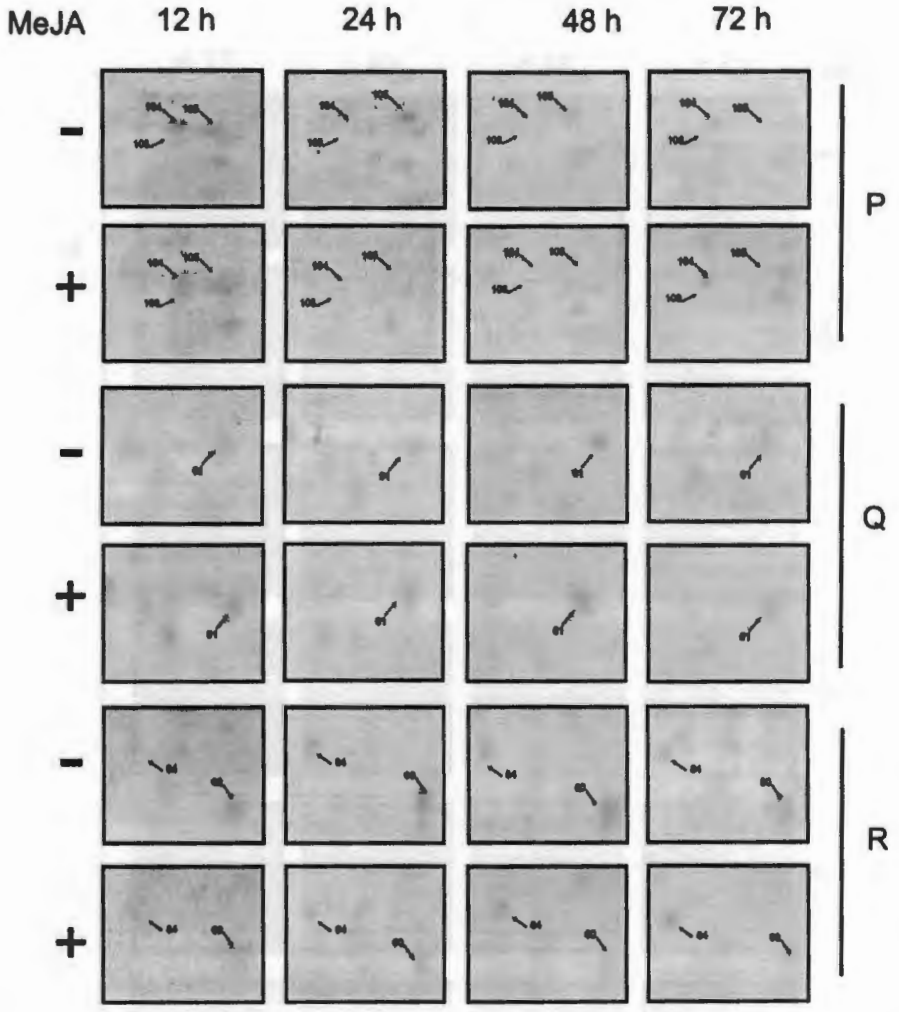


Figure S7. *Dynamic variations of root protein profiles by 2-DE. Three different regions (boxed in Figure 3.3A as P, Q and R). Total protein (750 μg) was separated by 2-DE in each time of treatment. Protein spots were visualized by staining with Coomassie Brilliant Blue R-250.*

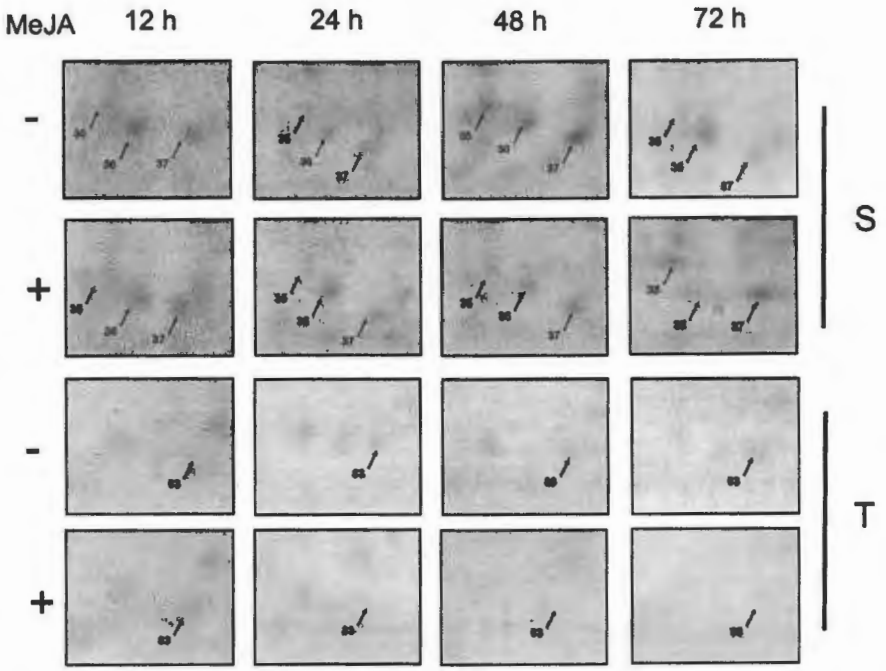


Figure S8. *Dynamic variations of root protein profiles by 2-DE. Three different regions (boxed in Figure 3.3A as S and T). Total protein (750 μg) was separated by 2-DE in each time of treatment. Protein spots were visualized by staining with Coomassie Brilliant Blue R-250.*

Chapter 4

4.1. GENERAL DISCUSSION AND CONCLUSION

Jasmonic acid (JA) and its volatile methyl ester (MeJA) are members of the hormonally active jasmonate family of compounds that are common throughout the plant kingdom. Since jasmonates (JAs) were first discovered in plants over 40 years ago, our understanding of their biosynthesis and physiological function has advanced considerably. The recent discovery of JAZ repressors in Arabidopsis and tomato has not only revealed new mechanical insights into JA signaling, but also reinforced the notion that signal-mediated degradation of repressors is a common theme used in plant hormone signaling (Thines *et al.*, 2007). The JAZ family contains at least 12 members in *A. thaliana*. So far, the involvement of at least three JAZ members (JAZ1, JAI3/JAZ3, and JAS1.3/JAZ10.3) with JA signaling has been demonstrated (Thines *et al.*, 2007). Many members of this gene family also show JA, wound, and herbivore induction (Thines *et al.*, 2007) although currently their roles in JA signaling are not clear. It is also possible that JAZ repressors may have additional roles in JA signaling. Despite observations of extensive interactions between JA and other hormonal signaling pathways, our knowledge on the molecular mechanisms involved in these interactions is also still rudimentary. Nevertheless, this complex interaction among signaling networks is a testament to the plant's ability to integrate diverse signals from multiple sources so expediently that a finely tuned output can be produced and thereby provide adaptation to its environment.

The concentration of JA *In planta* range about 15-20 ng g^{-1} FW (Mueller *et al.*, 1993; Creelman & Mullet, 1995; Creelman & Mullet, 1997) but when plants are treated with exogenous MeJA the levels of JA reach about 85.92± 0.91 ng g^{-1} FW (Howe,

2004; Pluskota *et al.*, 2007). This increasing concentration of JA is correlated with the diferential expression of several genes (Farmer *et al.*, 2003; Parani *et al.*, 2004; van Leeuwen *et al.*, 2007; Jung *et al.*, 2007) and the overaccumulation of different secondary metabolites(Vázquez-Flota *et al.*, 1994; Doughty *et al.*, 1995; Brader *et al.*, 2001; Zhao *et al.*, 2005; Hendrawati *et al.*, 2006). However, several of these studies have focused to the aerial parts of the plants, discriminating the below ground organs. Considering the implication of the roots in the integrative functionality of whole plants, such as mechanical support and water/nutrient uptake, synthesis, accumulation, and secretion of a diverse array of compounds (Flores *et al.*, 1999), it is very important to understand how the JAs are involved in the biological function of these organs. Because proteomics can reveal chemical complexity and biological dynamics, it provides functional information of the biochemical processes that are not accessible or predictable by other means(Chen & Harmon, 2006). Its objective has moved beyond simple cataloging to the study of functional and regulatory aspects of proteins such as comparative protein expression, postrancriptional modifications (PTMs), protein–protein interactions, subcellular localization, activities and structures (Chen & Harmon, 2006).

Before to carry out a proteomics approach, it was important to analyze specific parameters in our biological system. The first observation about the effect of MeJA in our hairy roots culture was the significant reduction of the dry weight. This first result agrees with the negative effect of JAs on the primary root growth of wild-type *A. thaliana* seedlings which was inhibited 50% when seedlings were grown on agar medium containing 0.1 μM MeJA (Staswick *et al.*, 1992). Cell suspension cultures of tobacco treated with 50 μM MeJA displayed similar growth rates with the control cells at the first 36, but after this initial period, cell growth was drastically reduced in jasmonate-treated cells when compared with no treated cells (Goossens *et al.*, 2003). This detrimental effect was always correlated with the induction of pathogenesis related compounds. In the case of *A.*

thaliana, the accumulation of vegetative storage protein (VSP) is observed wchich is considered as PRP(Staswick *et al.*, 1992; Farmer *et al.*, 1992). VSP appears to function as at temporary nitrogen store that accumulates during vegetative growth (Staswick, 1992). On the other hand, the tobacco cell suspension treated with MeJA overacumulation of nicotine was detectable after 12 h and reached rapidly its maximum level at the 48 h (Goossens *et al.*, 2003). Interestingly, similar results were observed in the preset work where the reduction in the dry weight of the hairy roots (Fig. 2.2) is correlated with the differential accumulation of TIAs (Fig. 2.3) (Ruíz-May *et al.*, 2008). In addition, differential secretion of TIAs was also observed in the cultures of hairy roots treated with MeJA (Fig. 2.4) (Ruíz-May *et al.*, 2008). This is the first report (Chapter II) which indicates the differential secretion of TIAs by MeJA treatment. Previous research suggested that secondary metabolites in root exudates are critical in specialized associations between plants and individual species of soil microbes ranging from mutualistic to pathogenic. For example, *Rhizobium* spp. are bacterial symbionts of legumes that are responsible for nitrogen fixation, and communication between the two organisms is mediated in part through root-secreted flavones (Broeckling *et al.*, 2008). However, the involvement of TIAs exudates by hairy root in some symbiotic interaction are still unknown. In the same scenario, deformation in the calyptra of the hairy roots was observed with scan microscope (Fig. 3.2) (Ruiz-May et al, manuscript in preparation). No report in the literature has been presented which indicates the above observation about the calyptra, so far. But it might suggest that this deformation contributed in some extend to the inhibition of roots growth.

Evidently MeJA induced a marked change in the metabolic fate of hairy roots cultures (Vázquez-Flota *et al.*, 1994; Ruíz-May *et al.*, 2008). To test this hypothesis, a proteomics analysis was carried out using a time course assay with 100 µM MeJA. This concentration induced good efficiency in the accumulation and secretion of TIAs without severe negative effect on the growth

of the hairy roots. Two-dimensional gel electrophoresis (2-DE) revealed several differentially expressed proteins in hairy roots treated with MeJA. Using quadruple-time of flight (Q-TOF) mass spectrometry analysis of diferential proteins lead to the indentification of 58 proteins belonging to eleven functional categories.

Proteins involved in carbohydrate metabolism (21%), cell growth and organization (10%), energy (7%) and cell cycle (3%) were highly repressed. In seedlings of rice similar results were observed with a proteomics approach where the repression of similar proteins was correlated with the inhibition of the growth of seedling roots (Cho *et al.*, 2007). Moreover, cell suspension cultures of tobacco treated with 200 μM of MeJA were obstructed in G2 phase, impairing the G2 to M transition in the cell cycle (Pauwels *et al.*, 2008). In agreement with the differential accumulation of TIAs (Fig. 2.3) in hairy roots, proteins involved in the secondary metabolites (7%) were highly expressed. Moreover, proteins involved in amino acid metabolism (12%), protein modification and chaperons (12%) were also highly expressed.

The repression of the glycolysis, diminution of ATP production and the obstructed of cell cycle could directly correlate with the inhibition hairy roots growth in our system (Fig. 2.2). Two global changes in the protein expression were observed in the hairy roots treated with MeJA. The inhibition of growth correlated with down regulation of primary metabolism which at least in part is mediated by the imposed G2 arrest of cell cycle progression (Pauwels *et al.*, 2008). On other hand induction in the accumulation and secretion TIAs correlated with the high expression of enzymes from secondary metabolism of plants such as DXR (*EC* 1.1.1.267; Table 3.1), peroxidase 1 (spot 24; EC 1.11.1.7) and flavonoid OMT (spot 31; EC 2.1.1.150). It is suggested that both behaviors are concomitantly regulated (Goossens *et al.*, 2003; Pauwels *et al.*, 2008; Zhang & Xing, 2008). The shikimate pathway links carbohydrate metabolism to the synthesis of aromatic amino acids (phenylalanine, tyrosine

and tryptophan), which can in turn act as precursors for various primary and secondary metabolites. The pathway begins with two intermediates of carbohydrate metabolism, phosphoenolpyruvate (PEP) (from glycolysis) and erythrose 4-phosphate (E4P) (from the pentose phosphate pathway), which are converted to chorismate in a sequence of seven metabolic steps (Roberts, 1998; Herrmann & Weaver, 1999).

This thesis is the first systemic work about the effect of MeJA on underground plant organ correlating the differential accumulation and secretion of TIAs with the behavior at the proteome level. Interestingly our results are in agreement in some extend with the transcriptomic level (Goossens *et al.*, 2003; Suzuki *et al.*, 2005; Pauwels *et al.*, 2008). Several pathogenic related (PRP) proteins such as VPS, JR2 and PDF1.2 are considered protein markers induced by exogenous application of MeJA on leaves (Midoh & Iwata, 1996; Cho *et al.*, 2007; Campbell *et al.*, 2003). However, no differential expression of PRP was observed in the hairy roots treated with MeJA. The observed differences might be due to the time point of collection of root samples analyses, concentration of MeJA used for elicitation or a differential response by roots to MeJA. These results indicate that different organs of the plant (leaves and roots) respond differently to this growth regulator (Badri *et al.*, 2008).

This systematic study provides new and detailed information on the effect of MeJA on the morphological responses in undergound plant organs and molecular changes at protein level, by employing an elegant *C. roseus* hairy roots culture study. Application of 2-DE PAGE approach in combination with mass spectrometry resulted in the identification of numerous MeJA-responsive proteins. Recently, the induction of a differential secretion of proteins by hairy roots treated with MeJA (Ruiz-May et al, manuscript in preparation) was observed. The activity of the secreted enzymes, like chitinase, was up-regulated by MeJA treatment. It is possible that MeJA,

like the differential secretion of TIAs, also regulates the differential secretion of specific proteins into the rhizosphere.

CHAPTER 5

5.1 PERSPECTIVES

The emerging new technologies have provided efficient tools for understanding the mechanisms which regulate central plant process. In the present work the investigation of effect of MeJA on hairy roots at the proteome level led to the identification of important proteins. This is the first approach to tackle the involvement of JAs in the plant root biology. More work is needed to get a complete understanding of this process. Some perspectives are enlisted below.

1. Identification of more proteins whose expression is altered by MeJA treatment is necessary. The fractionation of proteins could improve the resolution of protein pattern. Staining assay with high sensibility compatible with mass spectrometric could provide additional tools for the identification of low abundant proteins.
2. Posttranscriptional modification in several of cases is a prerequisite for the functionality of proteins. For this reason the study of the phosphoproteome, the glycoproteome and other protein postraslational modifications will let to get a better understanding of the role of MeJA in roots.
3. A detailed analysis of the effects of MeJA on secondary metabolites exudation in novel species is a necessary step in the investigation of how MeJA regulates this process.
4. Identification and isolation of ABC transporters which are directly correlated with the induction of the secretion of secondary metabolites by MeJA treatment.

5. Integrative study of the crosstalk of signal pathway of MeJA with other growth regulator such as salicylic acid, abscisic acid and ethylene.
   JD

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