Methyl Jasmonate Induces ATP Biosynthesis Deficiency and Accumulation of Proteins Related to Secondary Metabolism in Catharanthus roseus (L.) G. Hairy Roots

Elieel Ruiz-May1,4, Clelia De-la-Peña2,5, Rosa M. Galaz-Ávalos1, Zhentian Lei3, Bonnie S. Watson3, Lloyd W. Sumner3 and Víctor M. Loyola-Vargas1,*

1Unidad de Bioquímica y Biología Molecular de Plantas, Centro de Investigación Científica de Yucatán, Calle 43 No. 130, Col. Chuburná de Hidalgo, CP 97200, Mérida, Yucatán, México
2Department of Horticulture and Landscape Architecture and Center for Rhizosphere Biology, Colorado State University, Fort Collins, CO 80523, USA
3The Samuel Roberts Noble Foundation, Plant Biology, 2510 Sam Noble Parkway, Ardmore, OK 73401, USA
4Present address: Unidad de Biotecnología, Centro de Investigación Científica de Yucatán, Calle 43 No. 130, Col. Chuburná de Hidalgo, CP 97200, Mérida, Yucatán, México
5Present address: Departamento de Plant Biology, Cornell University, Ithaca, NY 14853, USA
*Corresponding author: E-mail, vmloyola@cicy.mx; Fax, +52-999-981-3900
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Jasmonates are specific signal molecules in plants that are involved in a diverse set of physiological and developmental processes. However, methyl jasmonate (MeJA) has been shown to have a negative effect on root growth and, so far, the biochemical mechanism for this is unknown. Using Catharanthus roseus hairy roots, we were able to observe the effect of MeJA on growth inhibition, cell disorganization and cell death of the root cap. Hairy roots treated with MeJA induced the perturbation of mitochondrial membrane integrity and a diminution in ATP biosynthesis. Furthermore, several proteins were identified that were involved in energy and secondary metabolism; the changes in accumulation of these proteins were observed with 100 μM MeJA. In conclusion, our results suggest that a switch of the metabolic fate of hairy roots in response to MeJA could cause an increase in the accumulation of secondary metabolites. This is likely to have important consequences in the production of specific alkaloids important for the pharmaceutical industry.

Keywords: Catharanthus roseus • Hairy roots • Methyl jasmonate • Proteome • Secondary metabolites.

Abbreviations: ACN, acetonitrile; ADH, alcohol dehydrogenase; ADK, adenosine kinase isofrom 2S; BSA, bovine serum albumin; 2-DE, two-dimensional electrophoresis; DTT, dithiothreitol; DXR, 1-deoxy-D-xylulose-5-phosphate reductoisomerase; ET, ethylene; HR, hairy roots; IDA, information-dependent acquisition; JAs, jasmonates; JA, jasmonic acid; LC/MS/MS, liquid chromatography–tandem mass spectrometry; MeJA, methyl jasmonate; OMT, flavonoid O-methyltransferase; RT–PCR, reverse transcription–PCR; SA, salicylic acid; TCA, trichloroacetic acid; TIA, terpene indole alkaloid

Introduction

Signal molecules, such as salicylic acid (SA), jasmonic acid (JA), ethylene (ET) and hydrogen peroxide (H₂O₂), have been shown to play a crucial role in mediating the biotic stress response (Orozco-Cardenas et al. 2001, Halim et al. 2006). Among these signal molecules, JA and related molecules called jasmonates (JAs) are involved in several other processes, such as root growth inhibition, tuber formation, plant tendril coiling and touch, flower development, wounding response and senescence (Aerts et al. 1994, Creelman and Mullet 1997, Gantet et al. 1998, Rijhwani and Shanks 1998, Lee-Parsons et al. 2004, Wasternack 2007, Pauwels et al. 2008).

Although microarray technologies and metabolic profiles have been used to examine the role of JAs in the regulation of secondary metabolism, resulting in the establishment of gene-to-metabolite networks (Achnine et al. 2005, Rischer et al. 2006, Naoumkina et al. 2007), more information about the corresponding proteins involved in the signal response of JAs is needed.

It was observed several years ago that methyl jasmonate (MeJA) induces secondary metabolite accumulation in Catharanthus roseus (Aerts et al. 1994, Menke et al. 1999, Van der Fits and Memelink 2000, Van der Fits and Memelink 2001, Ruiz-May et al. 2009). Furthermore, MeJA induces the accumulation of specific gene products in higher plants, an accumulation that correlates with some physiological effects (Farmer and Ryan 1992). For instance, at picomolar concentrations, primary root growth inhibition was observed as one of the first physiological effects of MeJA in Arabidopsis thaliana (Staswick et al. 1992); at micromolar concentrations,
MeJA treatment of A. thaliana suspension cultures induced an early response in the genes that encoded the JA biosynthesis pathway and key regulators of MeJA responses (Pauwels et al. 2008). JA treatment in tobacco BY-2 cells prevents DNA replication, keeping the cells in the G1 stage (Swiatek et al. 2002). Some works suggest that plant growth inhibition is linked directly to a decrease in the proteins related to energy metabolism, such as ATP synthesis (Cho et al. 2007). This detrimental effect has been correlated with the differential expression of different proteins (Farmer et al. 1992, Staswick et al. 1992). For instance, the induction of the vegetative storage proteins and proteinase inhibitors has been correlated with MeJA treatment in leaves (Farmer et al. 1992, Staswick et al. 1992). In C. roseus, it was found that the transcription factors Octadecanoid-Responsive Catharanthus AP2-domain (ORCA1, ORCA2 and ORCA3) are some of the most important proteins up-regulated by MeJA (Menke et al. 1999, Van der Fits and Memelink 2000). This transcription factor regulates multiple genes involved in primary and secondary metabolism, including several terpene indole alkaloid (TIA) biosynthetic genes (Aerts et al. 1994, Menke et al. 1999, Van der Fits and Memelink 2000, Van der Fits and Memelink 2001). Although numerous reports show changes in the protein profiles of leaves from different plant species in response to MeJA (Herrmann et al. 1989, Rakwal et al. 1996, Rakwal and Komatsu 2000, Cho et al. 2007), it is not known if and how the proteins change in roots. It is clear that important information is available about the genes expressed in the presence of MeJA. However, the biological role of the proteins is very important and needs to be addressed. In the present study, we conducted a systematic proteomics analysis of hairy roots (HR) of C. roseus exposed to MeJA to better understand the effects on the perturbation of mitochondrial integrity, which correlates with a reduction in the synthesis of ATP and the accumulation of specific alkaloids.

**Results**

**MeJA induces growth inhibition and oxidative burst in HR**

In order to know whether MeJA has any effect on HR, the roots were incubated in the presence of 10, 100 and 250 μM MeJA. It was observed that MeJA has a dramatic effect on root growth even at the lowest concentration (10 μM; Fig. 1a). When HR were exposed to the highest concentration of MeJA (250 μM), a reduction of 28% in the DW compared with the control at 72 h was observed. On the other hand, the reduction in DW was directly correlated with the perturbation of the membrane integrity in the root cap (Fig. 1b). The root cap of the control and with 10 μM MeJA showed no damage at 72 h. However, cell death was visualized in the root cap treated with 100 and 250 μM MeJA after 48 and 72 h, with the loss of cells being more dramatic with the treatment with 250 μM MeJA.

Both the reduction in DW and the perturbation of the cell membrane integrity suggest some kind of damage in the root cap of the HR after treatment with MeJA (Fig. 1). Scanning electron microscopy revealed a clear perturbation of the cell organization in the root caps treated with MeJA (Fig. 2). The cell disorganization was clearly visualized even with the lowest concentration of MeJA (10 μM) at 48 and 72 h, and some areas of cell layers from the root surface were detached (Fig. 2b, f). It was also observed that with 100 μM MeJA treatment, increasing zones of the root cap were detached (Fig. 2c, g). In the presence of the highest concentration of MeJA (250 μM), dramatic disorganization of many areas of the root cap was observed and several layers of cells were separated from the root cap surface (Fig. 2d, h). This cell disorganization might account for the reduction of DW (Fig. 1a) and the cell death observed (Fig. 1b).

**Proteome analysis of HR treated with MeJA**

In order to determine the proteins that are differentially changed following treatment with a moderate concentration of MeJA, we performed proteomics analysis on HR treated with 100 μM MeJA at different time points (Figs. 3, 4). It was observed that this concentration of MeJA was optimal because it had an effect on HR growth without disturbing the root cells excessively. The HR were analyzed by two-dimensional electrophoresis (2-DE) in a time course of 12, 24, 48 and 72 h after treatment with 100 μM MeJA, and clear differences were observed between 24 and 48 h (Figs. 3, 4; see the Materials and Methods). From the 108 proteins excised, 58 proteins were identified, and proteins with a MASCOT score >60 were significant at the P < 0.05 level (Table 1). The identified proteins belong to nine functional categories (Table 1). The majority of the HR proteins were classified as relating to carbohydrate metabolism, amino acid metabolism, cell growth and organization, protein modification and chaperones, energy and secondary metabolism. These proteins represented 69% of the total identified proteins in the HR. Here, we have simplified the analysis of the proteins by describing them using a functional category classification.

**Carbohydrate metabolism**

Twelve identified proteins belong to carbohydrate metabolism (Table 1). These proteins changed with time; however, it was observed that most of these changes happened at 48 h. Most of these changes were in proteins that decrease their accumulation in the presence of MeJA. The proteins that did not change at 48 h were alcohol dehydrogenase (ADH, spot 87; EC 1.1.1.1), fructokinase (spot 91; EC 2.7.1.4) and NADPH-specific isocitrate dehydrogenase (spot 107; EC 1.1.1.42). On the other hand, only isocitrate dehydrogenase (spot 68; EC 1.1.1.42) increased in the first 48 h of treatment with MeJA (Table 1).

**Amino acid metabolism**

From the 58 identified proteins, there were seven proteins related to amino acid metabolism, six of which were identified as S-adenosylmethionine synthetase (spots 15, 26, 34, 71 and...
Surprisingly, no protein was changed after 12 h of treatment with MeJA. The first changes were observed after 24 h, with an increase in spots 26, 71 and 98. After 48 h, spots 32, 34, 37 and 98 were reduced.

### Protein modification and chaperones

Seven proteins were identified from this category [chaperonine subunit β (spot 4), 20S proteasome α6 subunit (spot 33), heat shock protein 70 (spot 41), heat shock cognate 70 kDa protein 2 (spot 42), putative β-3 proteosome subunit (spot 49), proteosome subunit α type-5 (spot 83) and putative cysteine proteinase RD21A precursor (spot 92)]. From these proteins, only spots 42 and 92 were increased at 48 h (Table 1).

### Cell growth and organization

Most of the proteins belonging to cell growth and organization were reduced after 48 h with MeJA (Table 1). These proteins are the reversibly glycosylated polypeptides (spot 99; Fig. 4), xylan 1,4-β-xylosidase (spot 1; EC 3.2.1.37; Fig. 3) and actin protein (spots 2, 17 and 18). However, only spot 75, identified as actin, increased 72 h after MeJA treatment. These proteins are an important determinant in cytoplasmic streaming, cell shape determination, cell division, organelle movement and extension growth (McElroy et al. 1990). The change in the expression pattern of these proteins could be made possible by the active cell disorganization observed in the root cap of HR (Fig. 2).

### Cell cycle

In this category only two proteins were identified, a translationally controlled tumor protein homolog (spot 84; Figs. 3, 4, Table 1) and an adenosine kinase isoform 2S (ADK; spot 60; EC 2.7.1.20; Figs. 3, 4). However, only spot 84 changed at 24 h after MeJA treatment. The ADK is a modulator of root cap growth.
morphogenesis and gravitropism (Young et al. 2006). These results could be directly correlated with the cellular disorganization of the root cap of HR by MeJA (Fig. 2).

Secondary metabolism

In this category we observed the most changes in the accumulation of the proteins, and most of them happened upon early exposure to MeJA. It was possible to observe an increase in the accumulation of the proteins 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR; spots 20 and 21; EC 1.1.1.267; Figs. 3, 4; Table 1) and flavonoid O-methyltransferase (OMT; spot 31; EC 2.1.1.6; Figs. 3, 4) at 12 h, while peroxidase 1 was differentially accumulated at 12 and 72 h (spot 24; EC 1.11.1.7; Figs. 3, 4; Table 1) in the HR treated with MeJA.

Energy

In proteins related to energy, it was observed that the most changes were found at 24 and 48 h after exposure of HR to MeJA. For instance, ATP synthase (spots 6 and 7; EC 3.6.3.14; Fig. 5) and ATP synthase β-subunit mitochondrial precursor (spot 10; EC 3.6.1.34) were decreased at 48 h, while spot 7 increased at 24 h, but by 48 h the accumulation of this protein was low. Some of these proteins play a role in the general homeostasis of the cellular nucleoside triphosphate pools.

The other remaining functional categories correspond to a minor number of proteins that were identified in this study and most of them did not show important changes (Figs. 3, 4; Table 1).
Mitochondria: compelling target of MeJA signal

We clearly observed that the presence of MeJA involved an important reduction in the accumulation of proteins related to energy (Table 1); therefore, we wondered whether the mitochondrion is a target for the MeJA signal (Fig. 6). The ratio of Cyt c oxidase activity before and after the addition of 0.05% Triton X-100 (a non-ionic detergent) provides an approximation of the mitochondrial integrity (Millar et al. 2001).

In the first 40 h, the activity of Cyt c oxidase in the isolated mitochondria was constant in the absence or presence of Triton X-100 (Fig. 6a). However, MeJA slightly increased the activity of Cyt c oxidase in the HR in the absence of Triton X-100 at 48 h. Moreover, at the last time point of induction (72 h), the activity of Cyt c oxidase was significantly increased in the HR treated with MeJA in both the absence and presence of Triton X-100 (Fig. 6a). Because of the Cyt c oxidase location, it is possible to suggest that the membrane integrity of mitochondria was perturbed in the cultures of HR treated with MeJA. Interestingly, the perturbation of the mitochondrial membrane integrity was directly correlated with a decrease in ATP production in this organelle (Fig. 6b). The reduction of ATP production became evident after 48 h of treatment with MeJA, which also correlated with the decrease of the proteins related to energy (Table 1). However, by 72 h after treatment, a significant diminution of ATP production was observed (Fig. 6b). At this point, the mitochondrial ATP accumulation in HR treated with MeJA was 23.96 μg g⁻¹ FW, while in the control it was 39.71 μg g⁻¹ FW. These results correlate with the proteomics data, where the treatments with MeJA showed changes in

Fig. 4 Dynamic change of two-dimensional PAGE of total HR proteins after MeJA treatment. A representative proteomic map shows root tissue proteins from *C. roseus* HR tissues alone (control) or treated with 100 μM MeJA at 48 and 72 h. A 750 μg aliquot of protein was used. 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. The red arrow indicates the proteins that do not change, the blue arrow indicates the increment of protein intensity, and the green arrow indicates the reduction of intensity of proteins after the treatment with MeJA.
Table 1 List of the identified proteins differentially expressed in *Catharanthus roseus* hairy roots after exposure to 100 μM MeJA

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<th>S^#</th>
<th>P_α</th>
<th>MM (kDa)</th>
<th>pI</th>
<th>Organism identified under PAN</th>
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<th>24 h^T</th>
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<th>72 h^T</th>
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**Cell growth and organization**

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Corresponding spot numbers are indicated in Figs. 3 and 4.

\(^a\) MM, molecular mass (kDa); pl, isoelectric point.
\(^b\) Protein accession number (NCBI).
\(^c\) Mascot MOWSE score.
\(^d\) Number of peptides matched to the protein.
\(^T\) Time.
proteins related to energy metabolism and secondary metabolites (Fig. 5; Table 1). Also, these results correlate to some extent with the inhibition of the metabolic proteins and root growth inhibition (Figs. 1a, 3, 4).

**Discussion**

Because roots are a very important part of the plant, it is imperative to try to understand how this tissue responds when it is exposed to signal molecules such as MeJA. The present work provides new information about the biochemical and morphological alteration that MeJA treatments exert in HR cultured in vitro. The HR of *C. roseus* treated with MeJA was evaluated using biochemical and proteomics approaches; it was detected and identified most of the MeJA-responsive proteins in HR (Table 1). With the results shown in this work, we were able to reconstruct a proteomics-based metabolic pathway for HR cultures treated with MeJA (Fig. 7), which provided a better understanding of the MeJA response in HR.

The first physiological effect observed in HR after the exposure to 100 μM MeJA was the inhibition of root growth (Figs. 1b, 2). This inhibition was caused by the death of the cells. We evaluated cell membrane integrity by using the exclusion dye Evans blue, which leaks through ruptured membranes and stains the contents of dead cells (Baker and Mock 1994). The inhibition of the growth produced by JAs was discovered by two groups, one in Japan using Artemisia absinthium

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**Fig. 5** Representative gel section of the proteins involved in energy metabolism. The arrows and number indicate the identified proteins at the specific time of treatment. The dashes (−) indicate the control treatment and (+) indicate the treatments with 100 μM MeJA. 6, ATP synthase β-subunit; 7, ATP synthase subunit β mitochondrial precursor; and 104, nucleoside diphosphate kinase.

**Fig. 6** MeJA effect on mitochondria in HR. Analysis of mitochondrial integrity by Cyt c oxidase assay (a) and mitochondrial ATP production of HR (b). The white column and the column with a medium diagonal line pattern correspond to control and 100 μM MeJA treatment in the absence of Triton X-100, respectively; the column with a dense diagonal line pattern and the black column correspond to control and 100 μM MeJA treatment in the presence of 0.05% Triton X-100, respectively. The absence (filled squares) or presence of MeJA (filled circles, 100 μM) is denoted. Error bars represent ± SE (n = 3). An asterisk represents the statistical significance of mean differences at a given time by Turke’s test (*P ≤ 0.05; **P ≤ 0.01). The experiment was carried out three times.
(Ueda and Kato 1980) and the other in Germany working on Vicia faba (Dathe et al. 1981). Later, the same effect was described in A. thaliana (Staswick et al. 1992). More recently, similar observations were made in cell suspension cultures of tobacco (Goossens et al. 2003) and in roots of rice seedlings (Cho et al. 2007). However, until today it was not clear how the JAs produce this inhibitory effect. The inhibition of growth was thought to be linked to primary metabolism events, such as respiration. There is some evidence that suggests that some of the mitochondrial functions are targeted by MeJA (Zhang and Xing 2008). Our results suggest that the energy production machine is one of the prominent targets of the JAs pathway signal in plant cells, as has been recently suggested by Cho et al. (2007). Indeed, it is possible to demonstrate a drastic diminution in the biosynthesis of ATP in the treatments with MeJA (Fig. 6b) that correlates with the perturbation of the integrity of the mitochondrial membrane (Fig. 6a). Previous reports have shown that MeJA causes a series of alterations in mitochondrial dynamics, including the cessation of mitochondrial movement, the loss of mitochondrial transmembrane potential and the morphological transition and aberrant distribution of mitochondria followed by a subsequent cell death (Zhang and Xing 2008). Also, it has been found that MeJA strongly reduces the oxygen uptake during germination in isolated cotyledons (Corbineau et al. 1988).

A high concentration of MeJA induces direct perturbation of cancer cell mitochondria, leading to the release of Cyt c and eventual cell death (Flescher 2005). We found that the activity of Cyt c oxidase was significantly increased in the HR treated with MeJA (Fig. 6a). On the other hand, it was demonstrated...
that JAs bind to hexokinase (EC 2.7.1.1) and detach it from both the mitochondria and its mitochondrial anchor (Flescher 2005).

Biochemical studies suggest that hexokinase isozymes differ in their location within plant cells (Miernyk and Dennis 1983, Singh et al. 1993, da-Silva et al. 2001, Giegé et al. 2003, Heazlewood et al. 2004, mari-Weissler et al. 2006, mari-Weissler et al. 2007). As in humans, it could be that MeJA is targeting the hexokinase isozyme attached to the mitochondria and at the same time, probably as a consequence of this first effect, the mitochondrial membrane is disrupted, producing the dysfunction of the mitochondria (Flescher 2005).

MeJA is involved in the signal transduction pathway, leading to the response of the plant to different environmental signals (Gfeller et al. 2006). One of these responses is the biosynthesis of proteins and small molecules belonging to secondary metabolite compounds. For several years, JAs have been used as elicitors of secondary metabolism in many plant tissue culture species, in particular C. roseus (Guillon et al. 2006, Loyo-Vargas et al. 2007). However, TIA genes exhibit significant variation in the magnitude and timing of induction by MeJA (Wei 2010), Jacobs et al. (2005), using a proteomics approach in C. roseus suspension culture, detected 88 proteins spots from which 58 were identified by mass spectrometry (MALDI-MS/MS). Among these proteins, only strictosidine synthase and tryptophan synthase that are involved directly and indirectly in alkaloid biosynthesis were detected.

In the present research, we were able to identify 54 protein spots by liquid chromatography–tandem mass spectrometry (LC/MS/MS) that included the flavonol 3′,5′-O-methyltransferase (spot 31; EC 2.1.1.149; Table 1 (Cacace et al. 2003)) identified in C. roseus cell cultures. This gene is expressed in roots and leaves of wild-type C. roseus plants, as well as in the HRcultures (Fig. 8). It was found that the expression of the gene increased with MeJA treatment in comparison with the HR control, which supports the abundance of the protein in MeJA treatment (spot 31; Table 1). We used A. thaliana as a negative control to confirm that this gene is only expressed in C. roseus. The understanding of the effects, this gene beneficial or deleterious, on secondary metabolism biosynthesis produced by the use of elicitors such as MeJA could lead to improvements in the biotechnological production of some important secondary metabolites.

As DXR (EC 1.1.1.267) is the first committed catalyzed step of the non-mevalonate pathway (MEP) (Lichtenthaler et al. 1997), the increase of this enzyme due to MeJA in HR (Figs. 3, 4; spots 20 and 21; Table 1) could improve the production of secondary metabolites important for the pharmaceutical industry. The elimination of 2,4-D from the culture medium produces the accumulation of crd transcripts from day 4 onward, at the same time that the accumulation of TIA in C. roseus suspension cultures occurs, showing that Crdr is up-regulated in C. roseus cell suspension culture under induction conditions (Veau et al. 2000). Previously, we observed the accumulation of TIA in different in vitro cultures treated with MeJA (Ruiz-May 2008) and now we are able to suggest that not only the incremental increase in the level of transcript (Veau et al. 2000), but also the increment of the DXR protein after the treatment of the HR with MeJA (Fig. 3; spots 20 and 21; Table 1) is part of the mechanism of the TIA increment. We found that the DXR gene is expressed at the same level in leaves and roots of C. roseus wild-type plants, as well as in the C. roseus HR in all the treatments, except in A. thaliana leaves where no expression was detected (Fig. 8). These data not only support the fact that the biosynthesis of the terpene moiety of monoterpene indole alkaloids in C. roseus HR is carried out through the non-mevalonate pathway, but also that there is a post-transcriptional control that led to the increase of the protein present in the cells.

There is a decrease in basic metabolism, including a decline in several of the proteins that belong to carbohydrate metabolism, in the presence of MeJA (Figs. 3, 4; spots 11, 91, 46, 65, 60 and 90). It is possible that this disruption of primary metabolism could lead, along with the direct effect of the MeJA on the mitochondria, to the observed decrease in growth and ATP production. The increase in HSC70 (Figs. 3, 4; spot 42) could also be a component of this response, since it has been proposed that the overexpression of these proteins contributes to optimum plant growth, development and thermotolerance. Also, it has been observed that the overexpression of these heat shock proteins appears to be deleterious to plant viability, growth and development (Sung and Guy 2003).

Another important response to the presence of MeJA is the expression of proteins involved in the potential to control pest and insect attack (Lim et al. 1996, Zhang et al. 1999, Vancanneyt et al. 2001). We found that some of these proteins, such as the ADHs (spots 87 and 89), increased with the treatments with MeJA. It is well known that ADH overexpression...
alters the production of C6 volatiles (Bate et al. 1998). Interestingly, there is a strict correlation with the release of C6 volatiles and the increase of JA biosynthesis (Engelberth et al. 2004). JA and C6 volatiles share an intermediate, 13-hydroperoxy-linolenic acid, the product of the action of lipoygenase on linolenic acid in MeJA biosynthesis (Gatehouse 2002). These changes in the expression of the proteins previously described illustrate the switch in the metabolism status of the HR after treatment with MeJA.

On the other hand, the differential expression of ADK (spot 60), the translation initiator factor SA-2 (spot 61) and 14-3-3-like protein (spot 93), which are related to the modulation of root cap morphogenesis, programmed cell death and several stress responses (Kidou et al. 1993, Li et al. 2004, Thompson et al. 2004, Young et al. 2006), coincided with the change in the morphology and membrane damage of the root cap observed in our system (Figs. 1, 2).

In summary, the transient response that we observed in the expression of some proteins in this study and the TIA metabolites (Ruiz-May et al. 2009) is similar to that found by Peebles et al. (2009) for TIA transcripts and metabolites when C. roseus HR are challenged by JA. This finding suggests that in the presence of exogenous elicitors, such as MeJA, C. roseus HR closely control the TIA biosynthetic pathway and rapidly bring the system back to the basal level.

Materials and Methods

HR culture and elicitation assay

The J1 line of C. roseus HR was obtained by infecting the roots with Agrobacterium rhizogenes strain 1855 pBI 121.1 (Ciau-Uitz et al. 1994). This line was maintained in half-strength B5 medium (Gamborg et al. 1968) supplemented with 3% (w/v) sucrose, incubated in the dark at 100 r.p.m. at 25°C for 15 days. MeJA solutions were prepared by diluting the MeJA stock (Aldrich, 95% purity) in absolute ethanol (J.T. Baker, 99.8%). Concentrations of 10, 100, and 250 μM MeJA were added to a 15-day-old HR culture and for the ethanol control absolute ethanol alone was used. Root tissue was collected at 12, 24, 48, and 72 h after elicitation with MeJA. The MeJA response is not produced only at the site of exposure; in fact, the MeJA signaling is systemic. For instance, Farmer et al. (1992) found in tomato leaves that mRNAs and proteins such as proteinase inhibitor proteins I and II were accumulated in distal leaves at similar levels to those leaves that were exposed to MeJA or jasmonic acid.

FW and DW determination

HR treated with MeJA were collected and weighed for FW determination. For DW, the roots were frozen at −80°C and freeze-dried. After the total elimination of water was achieved, the roots were weighed. Each sample was tested in triplicate. The experiment was repeated twice.

Membrane integrity

The plasma membrane integrity was measured by Evans blue staining (0.025% (w/v) Evans blue in 100 μM CaCl2, pH 5.6) for 10 min. Stained HR were washed three times with 200 ml of 100 μM CaCl2 (pH 5.6) until the dye was no longer eluted from the roots (Yamamoto et al. 2001). The stained roots were examined on a Leica M2FL III stereo microscope and the images were recorded with an integrated Leica DFC320 digital camera.

Cyt c oxidase assay

Cyt c oxidase activity was measured in 20 mM MES/NaOH (pH 5.9) buffer containing 1% (w/v) Tween-80 and 10 μM ascorbate reduced Cyt c (horse heart, Sigma type III). The oxidation of the Cyt c was followed by a decrease in absorbance at 550 nm, and an extinction coefficient for Cyt c of 18.7 mM⁻¹ cm⁻¹ was used to calculate the activity (Hawkesford et al. 1989).

Electron microscopy

The HR tissues were fixed in 1% glutaraldehyde, 4% formaldehyde in 50 mM sodium phosphate buffer at pH 7.2 for 3 h, and rinsed for 30 min 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 E5100) and plated with gold using 30 mA for 60 s at 120 mTorr, until a layer of 150 Å thick 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.

2-DE separation

Frozen HR were ground in liquid nitrogen with a mortar and pestle. The resultant material was homogenized with the extraction buffer [7 M urea, 2 M thiourea, 4% (v/v) NP-40, 1% (v/v) dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM EDTA, 40 mM Tris–HCl, 1% (v/v) ampholytes mixture (pH 3–10) and 0.05% polyvinyl pyrrolidone (PVPP)]. The homogenates were centrifuged at 15,000 g for 15 min. The supernatant was precipitated by cold 10% (v/v) trichloroacetic acid (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 g), the samples were washed three times with 10 ml of 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 lysis buffer containing 7 M urea, 2 M thiourea, 2% (v/v) NP-40 and 2% (v/v) ampholytes mixture [1.6% (v/v) pH 5–7 and 0.4% (v/v) pH 3–10]. This mixture was agitated thoroughly and then centrifuged (15 min, 16,000 g). The supernatant was recovered and stored in aliquots at
were transferred to polypropylene 96-well plates for further analysis. Coomassie was removed (Watson et al. 2003). These gel plugs were overlaid with buffer consisting of 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% (w/v) bromophenol blue and isoelectrically focused at 600 V for 5 h, 800 V for 12 h and 1,200 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) DTT, 30% glycerol (v/v) and 0.05% (w/v) bromophenol blue] at room temperature for 15 min. Next, the gels were placed on 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 de-stained the next day. Image analysis was performed visually and the changes observed were qualitative in nature. The 2-DE PAGE gel experiments were repeated at least three times to confirm reproducibility.

**In-gel trypsin digestion**

Protein spots were manually excised from the gel, washed twice with water for 15 min, and de-stained with a 1:1 (v/v) solution of acetonitrile (ACN) 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 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 bicarbonate, Roche Diagnostics). After rehydration for 30 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 for 13 h at 37°C and stopped by adding 15 μl of 10% formic acid. All peptide extract fractions were pooled, concentrated until dry and resuspended in a 50:50 (v/v) water–ACN solution containing a final concentration of 0.1% formic acid.

**LC/MS/MS**

Separations of the digest’s proteins were achieved using a nanoscale HPLC system (LC Packings) consisting of an autosampler (Famos), a pre-column switching device (Switchos) and an HPLC pump system (Ultimate). Samples (5 µl) were loaded onto a C18 pre-column (0.3 mm inner diameter × 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 pre-column and separated on a nano analytical C18 column (75 μm internal diameter × 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) maintained at a voltage of 2,400 V. Time-of flight mass spectrometry (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.

**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.) search engine (Perkins et al. 1999, Creasy and Cottrell 2002) with the following settings: a mass tolerance of 0.05 p.p.m., one trypsin missed cleavage allowance 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.

**Mitochondria isolation**

Mitochondria were isolated according to Arron et al. (1979) and Millar et al. (2001). In brief, HR were ground with liquid nitrogen and homogenized with the extraction buffer [0.4 M mannitol, 5 mM EGTA, 50 mM sodium pyrophosphate-KOH (pH 7.8), 10 mM cysteine, 0.5% (w/v) bovine serum albumin (BSA) and 1% (w/v) PVP-40]. The homogenate was centrifuged at 1,500 × g for 5 min and the supernatant was centrifuged again at 18,000 × g for 15 min. The resulting pellet was PI.
washed twice in a standard wash medium [0.3 M sucrose, 10 mM MOPS-KOH, pH 7.2, and 0.1% (w/v) BSA] at 18,000 × g for 15 min. The crude organellar suspension was layered over a linear sucrose gradient [20–60% (w/v)] and centrifuged at 75,000 × g for 3 h. After centrifugation, a formed colored band was aspirated with a Pasteur pipet and washed with standard wash medium at 18,000 × g for 15 min. The resulting mitochondrial pellet was resuspended in wash medium containing 7.5% (v/v) ethylene glycol and stored at −80°C until use.

**ATP determination by HPLC**

ATP was extracted from the mitochondrial sample with 5 ml of 0.6 M perchloric acid in an ice bath for 1 min by the method of Yang et al. (2002) and then centrifuged at 6,000 × g for 10 min at 4°C. The supernatant was quickly neutralized to pH 6.5–6.8 with 1 M KOH solution and then allowed to stand for 30 min in ice to precipitate most of the potassium perchlorate, which was removed by centrifugation at 15,000 × g for 15 min. The supernatant was stored at −80°C prior to the analysis.

The HPLC method reported by Liu et al. (2006) was followed. In brief, the samples were chromatographed by continuous gradient elution on a 4.6 mm × 150 mm reverse phase, Zorbax Eclipse XDB, 5 μm particle size C18 column (Agilent Technology). The chromatographic system (Agilent series 1200) consists of quaternary G1311 A pumps connected to a G1329 A automatic sample injector. The injected samples (20 μl) were detected at 254 nm with the gold 168 diode array detector G1315B (Agilent Technology). The mobile phase A consisted of 60 mM K₂HPO₄ and 40 mM KH₂PO₄ dissolved in HPLC water and adjusted to pH 7.0 with 100 mM KOH, while mobile phase B consisted of 100% ACN. The flow rate of the mobile phase was 1.2 ml min⁻¹, while the injection volume was 20 μl. ATP in the samples was identified by comparison with the retention time of the standards, while the concentrations of ATP were determined using the external standard method. Data were expressed as means of three independent replicate determinations.

**RT–PCR**

Total RNA was extracted from 0.1 g of root and leaf tissue by using TRI reagent (Sigma) according to the manufacturer’s instructions. Reverse transcription reactions were performed in a 20 μl volume containing 1.5 μg of total RNA and 200 U of the SuperScript II Reverse Transcriptase (Invitrogen), according to the manufacturer’s instructions. The PCR conditions were as follows: 5 min at 95°C, followed by 28 cycles of 95°C for 40 s, 58°C for 40 s and 72°C for 90 s, and the final cycle of 72°C for 5 min. The following primers were used to determine gene expression in HR of C. roseus, root tips and leaves of C. roseus wild type and leaves of A. thaliana wild type: for O-methyltransferase (AV OMT) forward, 5'-TGTGGAAACAGTCCTTCCTCCA-3' and reverse, 5'-TGACGACCTACCTCCACC-3'; for 1-deoxy-D-xylulose-5-phosphate reductoisomerase (AF DXR) forward, 5'-GTCGTGAGCTGTAC-3' and reverse, 5'-TACAAGGGCCAGGGCTCAAAC-3'; and for UBQ forward, 5'-GACGGGCCGCCACCCTTGGGATTGA-3' and reverse, 5'-TCCCTGGATCTTGCCCTTGA-3'. The primers were designed using the FastPCR program (http://www.biocenter.helsinki.fi/bi/Programs/download.html) from highly conserved nucleotides in the 3' region [GenBank accession Nos. AY127569 (Cacace et al. 2003) and AF250235 (Veau et al. 2000), respectively].

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**References**


Methyl jasmonate effects in *C. roseus* hairy roots


