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Necessity of a Functional Octadecanoic Pathway for Indole Alkaloid Synthesis by *Catharanthus roseus* Cell Suspensions Cultured in an Auxin-Starved Medium

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The effect of methyl jasmonate (mJA), jasmonic acid and traumatic acid, derivatives of the octadecanoic pathway, on the production of alkaloids by cell suspension cultures of *Catharanthus roseus* L. (G) Don was investigated. Cells cultured in the presence of auxin (m-cells) did not accumulate alkaloids. The addition of exogenous mJA to m-cells restored the ability to produce alkaloids. In cells cultured in a 2,4-D-starved medium (p-cells), exogenous mJA greatly increased alkaloid production. Similar data were obtained for jasmonic acid. In contrast, traumatic acid had no effect on alkaloid production. The sensitivity of cell suspension cultures to exogenous mJA was restricted to the first four days of subculture corresponding to the active growth phase, whereas the alkaloid accumulation occurred only during the stationary phase of the subculture (days 6 to 10). When p-cells were treated with octadecanoic pathway inhibitors, the ability to produce alkaloids was strongly reduced. The addition of exogenous mJA always restored the ability to produce alkaloids. These data suggest that in response to auxin depletion, endogenous mJA could be produced and act by linking physiological events thus leading to alkaloid biosynthesis activation.

Key words: Alkaloid — Auxin — *Catharanthus roseus* — Methyl jasmonate — Octadecanoic pathway — Periwinkle.

Catharanthus roseus produces several valuable therapeutic indole alkaloids such as ajmalicine, used in the treatment of circulatory diseases, and vincristine and vinblastine which are powerful anti-tumor drugs. Alkaloid production has been extensively studied in *C. roseus* cell suspension cultures (Meijer et al. 1993) but the regulation of their biosynthesis remains unclear. Several studies stress that auxin, which is essential in vitro to overcome an unidentified rate-limiting step in cell division, is a powerful inhibitor not only of alkaloid biosynthesis in *C. roseus* cell suspension cultures (Morris 1986) but also of other secondary metabolites such as anthocyanins (Hall and Yeomann 1986). Alkaloid biosynthesis was induced by transferring *C. roseus* cells from a medium containing an auxin, 2,4-dichlorophenoxyacetic acid (2,4-D), to a 2,4-D-depleted medium. The inhibitory effect of auxin on alkaloid biosynthesis takes place principally during the first three days of the culture although the secondary metabolites accumulated only during the stationary phase of the culture (Arvy et al. 1994). Several studies have dealt with auxin signal transduction during the last few years (Napier and Venis 1995). However, the signal triggered by auxin depletion and its transduction are as yet unknown. Moreover, nothing is known about the way by which this signal acts positively on alkaloid accumulation in *C. roseus* cells.

An activation of monoterpenic indole alkaloid biosynthesis by jasmonates was reported in *C. roseus* and *Cinchona ledgeriana* seedlings (Aerts et al. 1994) and in cell suspensions of *Rauvolfia canescens* (Gundlach et al. 1992). In plant-pathogen interaction, jasmonates are powerful inducers of genes encoding pathogen protectants such as proteinase inhibitors (Herde et al. 1996), stress protectants such as osmotin (Lehmann et al. 1995), or enzymes such as phenylalanine ammonialyase and chalcone synthase or 3-hydroxy-3-methylglutaryl Coenzyme A reductase (HMGR) (Choi et al. 1994, Maldonado-Mendoza et al. 1994). In *C. roseus* the alkaloids stemmed from mevalonate biosynthesized by HMGR. The involvement of jasmonates has been suggested in a signal transduction pathway leading to plant defence activation in response to several stresses such as wounding, elicitor treatment or UV irradiation (Gundlach et al. 1992, Conconi et al. 1996). It has been shown that in response to elicitation, plasmalemma phospholipase A2 was activated to produce linolenic acid by cleaving membrane phospholipids (Chandra et al. 1996). Linolenic acid was metabolized into jasmonates through the octadecanoic pathway. These phytohormonal compounds were then able to activate plant defence genes (Sembdner and Parthier 1993). As the alkaloid biosynthesis is upregulated by auxin depletion in *C. roseus* cells and by exogenous jasmonate in *C. roseus* seedlings (Aerts et al. 1994) the possible existence of a functional link between these two signals was investigated. The hypothesis was formulated that the auxin depletion signal could be perceived as a stress by the cell suspension culture. In response to this

Abbreviations: AA, aristolochic acid; DIECA, diethyldithiocarbamic acid; mJA, methyl jasmonate; p-cells, cell suspension cultured in a productive medium without 2,4-D; PG, propyl gallate; m-cells, cell suspension cultured in a maintenance medium with 2,4-D; SA, salicylic acid; SHAM, salicylhydroxamic acid.

stress, the octadecanoic pathway could be activated in order to produce endogenous jasmonates which could take part in a cascade of physiological events leading to the stimulation of alkaloid biosynthesis. In order to test this hypothesis the effects of the following were investigated on alkaloid biosynthesis in cell suspensions cultured in the presence or absence of auxin: (i) exogenous jasmonates (ii) inhibitors of endogenous jasmonate biosynthesis.

Materials and Methods

Cells and culture conditions—All experiments were conducted on a cell suspension culture of *Catharanthus roseus* (L) G. Don strain C20, 2,4-D-dependent and cytokinin-independent. The cells were grown in 50 ml Gamborg B5 medium (Gamborg et al. 1968) containing 58 mM sucrose and 4.5 μM 2,4-D (maintenance medium, m-cells). Cultures were started by adding 5 ml of a 7-day-old stationary phase culture to 45 ml of fresh medium and were grown in the dark at 24°C on a rotary shaker at 100 rpm. Cells were also grown for one culture in a 2,4-D-free B5 medium (production medium, p-cells). Cells were harvested by filtration through a 30 μm nylon cloth under reduced vacuum and were washed with cold water. They were then deep-frozen, freeze-dried and weighed to determine the dry mass.

Reagent feeding—Diethyldithiocarbamic acid (DIECA), in water solution, was sterilized by filtration on a Syrfil MF filter (0.22 μM) and added at day 3 to the cell suspension to obtain a final concentration of 1 mM. Other reagents were dissolved in ethanol and fed to the cell suspension at day 3 to obtain a final concentration of 0.5 μM for aristolochic acid (AA), 400 μM for methyl jasmonate (mJA), 500 μM for traumatic acid and 1 mM for salicylic acid (SA), propyl galate (PG) and salicyl hydroxamic acid (SHAM).

Ajmalicine determination—Aliquots of 60 mg freeze-dried cells were used for alkaloid quantification. Ajmalicine analysis was performed as described by Mérillon et al. (1986).

Reproducibility—The results reported represent those from one experiment. It should be noted, however, that this experiment was replicated 4 times. Despite some inevitable variation, each replication produced similar results, thus supporting the conclusions of the present study.

Results

Effect of jasmonates on alkaloid accumulation—*Catharanthus roseus* cells (strain C20) grown in a maintenance medium containing 4.5 μM of 2,4-D (m-cells) did not accumulate measurable amounts of alkaloids. These cells accumulated ajmalicine if mJA was added at day 3 (Fig. 1A). This induction of ajmalicine production by mJA occurred in a dose-dependant manner when mJA was supplied from 100 μM to 1,000 μM. The maximum ajmalicine production, 200 μg (g DW)$^{-1}$, was obtained when mJA was fed at a final concentration of 400 μM in the growth medium. This rate of ajmalicine content remained half that observed when m-cells were diluted ten-fold with an auxin-free medium (p-cells) and cultured for one cycle in this

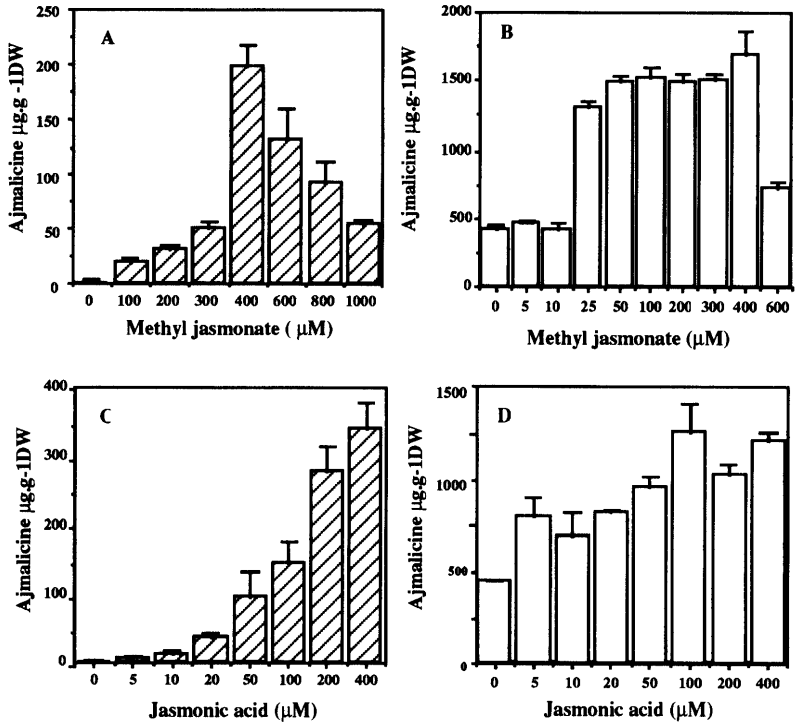

Fig. 1 Effect of jasmonates on ajmalicine amount in *Catharanthus roseus* cells (C20 strain) cultured in B5 medium containing 4.5 μM 2,4-D (m-cells, A and C) or lacking 2,4-D (p-cells, B and D). Methyl jasmonate (A and B) or jasmonic acid (C and D) were added on day 3 of culture. Cells were harvested on day 10 of culture. Each data point represents the mean (with SE) of 3 replicates.

medium (Fig. 1B). During this unique culture cycle, the growth expressed in DW or in DNA content was not significantly modified compared to that observed in m-cells (data not shown). When mJA was supplied at day 3 to p-cells, ajmalicine production increased to reach a plateau (Fig. 1B): ajmalicine production was about 1,200 to 1,700 μg $(g\ DW)^{-1}$ from a mJA concentration of 25 μM to 400 μM, which represented a three-fold increase in the ajmalicine content of p-cells.

Similar data were obtained when m-cells or p-cells were supplied with another jasmonate compound, jasmonic acid (Fig. 1C, D). In contrast, if cells were fed with a different linolenic acid bioactive subproduct (Fig. 2), traumatic acid, their ability to produce ajmalicine remained unchanged, even when cells were cultured in the absence or in the presence of auxin (data not shown).

Time course of alkaloid biosynthesis regulation by methyl jasmonate—In control p-cells, ajmalicine accumulation occurred at the beginning of the stationary phase (day 6 of the subculture). Feeding with methyl jasmonate did not modify qualitatively the time course of ajmalicine accumulation in p-cells. This time course was the same in m-cells fed with mJA (Fig. 3). When mJA at 400 μM was fed to m-cells at different times of the subculture, it appeared that its inductive effect on ajmalicine accumulation increased up to day 3 of the subculture and then decreased up to day 5 (Fig. 4A). This result stressed that cell sensitivity to the mJA signal was maximal on day 3 of the culture, corresponding to the active cell growth phase period. When mJA at 400 μM was fed to p-cells at different times of the subculture, its inductive effect on ajmalicine accumulation reached a plateau which remained constant for the first four days of subculture and then started to decrease (Fig. 4B).

Effect of octadecanoic pathway inhibitors on alkaloid biosynthesis—In order to test whether endogenous jasmonates were involved in alkaloid biosynthesis regulation in response to the auxin depletion signal, cells were cultured in the presence of different inhibitors of the octadecanoic biosynthetic pathway: aristolochic acid (AA), diethyldithiocarbamic acid (DIECA), propyl gallate (PG), salicylic acid (SA) and salicylhydroxamic acid (SHAM). These inhibitors were characterized by different action mechanisms. AA was inhibitor of phospholipase A2 (Chandra et al. 1996), SHAM and PG of lipoxygenase (Doares et al. 1995) and SA of hydroperoxide dehydrase (Pena-Cortes et al. 1993) (Fig. 2). In contrast DIECA rapidly and efficiently caused the chemical reduction of 13(S)-hydroperoxylinolenic, the product of the oxidation of linolenic acid by lipoxygenase. The efficient reduction of 13(S)-hydroperoxylinolenic acid to 13-hydroxylinolenic acid prevents its cyclization and subsequent conversion to jasmonic acid (Farmer et al. 1994). For each of these inhibitors, the con-

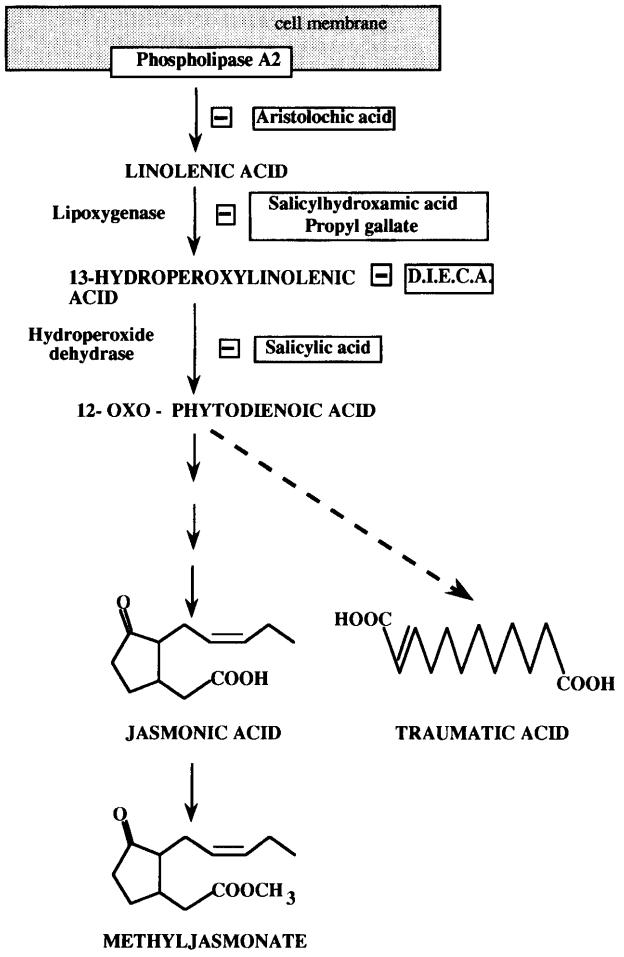

Fig. 2 Biosynthetic pathway of jasmonates and traumatic acid, and sites of action of the inhibitors used in this study.

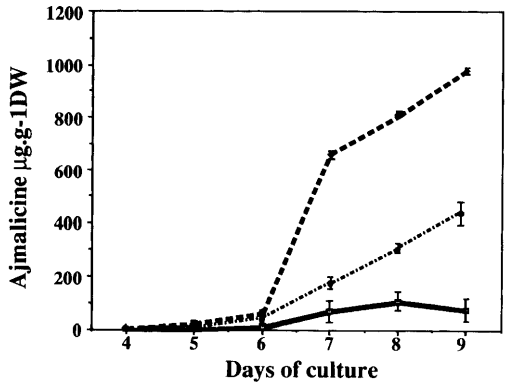

Fig. 3 Effect of feeding methyl jasmonate 400 μM on ajmalicine accumulation time course in *Catharanthus roseus* cells, strain C20. Methyl jasmonate was added on day 3 of culture. -⊡-, cells cultured in B5 medium containing 4.5 μM of 2,4-D. -■-, cells cultured in B5 medium lacking 2,4-D and methyl jasmonate. ---●---, cells cultured in B5 medium lacking 2,4-D. Each data point represents the mean (with SE) of 3 replicates.

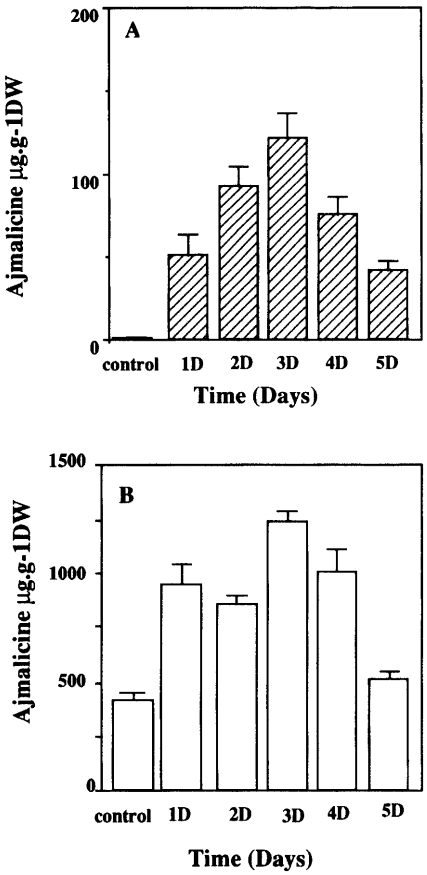

Fig. 4 Effect of methyl jasmonate feeding time on ajmalicine accumulation in *Catharanthus roseus* cells (C20 strain) cultured in a B5 medium containing 4.5 μM 2,4-D (m-cells, A) or lacking 2,4-D (p-cells, B). Methyl jasmonate was fed at a final concentration of 400 μM each day between the 1st and the 5th day. Cells were harvested on day 10 of culture. Each data point represents the mean (with SE) of 3 replicates. Control: cells cultured without methyl jasmonate.

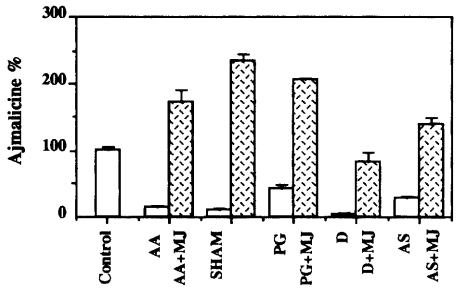

Fig. 5 Effect of octadecanoic pathway inhibitors and methyl jasmonate supply on ajmalicine amount in *Catharanthus roseus* cells (C20 strain) cultured in a B5 medium lacking 2,4-D (p-cells). Octadecanoic pathway inhibitors were added on day 3 of culture (□), methyl jasmonate (400 μM) was added 1 h later (⊠). Cells were harvested on day 10 of culture. Each data point represents the mean (with SE) of 3 replicates.

centration triggering an inhibition of alkaloid biosynthesis without effect on cell growth measured as dry weight was determined (data not shown). The impact of these inhibitors on ajmalicine production by p-cells was then tested. As shown in Fig. 5, when p-cells were supplied with these inhibitors of endogenous mJA biosynthesis at day 3, ajmalicine accumulation by cells was always reduced. If cells were cultured in the same conditions but with an exogenous feeding of mJA at 400 μM one hour after octadecanoic pathway inhibitor addition, cell capacity to accumulate ajmalicine was restored to a level at least similar to that observed for p-cells cultured in the absence of the octadecanoic pathway inhibitor.

Discussion

This study tested the action on alkaloid biosynthesis by *Catharanthus roseus* cell suspension cultures of three different physiologically active molecules issued from linolenic acid through the octadecanoic pathway (jasmonic acid, mJA and traumatic acid) (Fig. 2). Traumatic acid is, like jasmonic acid and mJA, a stress responsive compound which was first identified as an inducer of callogenesis (Strong and Kruitwagen 1967). In our system, traumatic acid used at different concentrations had no effect on alkaloid biosynthesis. In contrast jasmonic acid and its methyl ester were strong inducers of alkaloid synthesis indicating a specific action of jasmonates on this biosynthetic pathway. Jasmonic acid and mJA were able to overcome, at least partially, the auxin-induced inhibition of indole monoterpenic alkaloid biosynthesis in cell suspensions of *C. roseus*. In p-cells the ajmalicine accumulation induced by mJA is three times that observed in m-cells. These results are related to those reported by Ishikawa et al. (1994) in tobacco cells, the induction by mJA of Cathepsin D inhibitor gene was maximal in the absence of auxin. As the repression of mJA inducible expression of gene by auxin was released by aphidicoline, these authors suggested that mJA inducing expression is repressed during active cell division. In the C20 strain of *C. roseus*, no correlation between cell division and repression by auxin of mJA ajmalicine accumulation induction could be established as the time courses of DW and of DNA content in m-cells or in p-cells during the culture were not significantly different.

The sensitivity of cells to mJA was restricted to the first days of the culture in m-cells and in p-cells. In p-cells this remained constant at a maximal level during the first four days of the subculture and then began to decrease from the fifth day of the subculture. In m-cells this sensitivity increased to reach a maximum at day 3 and then decreased at day 5. These differences observed between p-cells and m-cells could be at least explained by the fact that in p-cells endogenous mJA could be produced in response to auxin depletion. In p-cells the ajmalicine content observed

could reflect the combined action of endogenous and exogenous mJA, or, on the other hand, a potentiation effect of auxin depletion signal on the sensitivity of cells to respond to mJA (Graham and Graham 1996). Nevertheless, this variation in the sensitivity of cell suspensions to mJA was reminiscent of the one described for *C. roseus* seedlings. In this latter experimental model mJA stimulated alkaloid biosynthesis but the sensitivity to mJA decreased progressively during the seven days following germination (Aerts et al. 1994). Authors have suggested that, when the initiation of alkaloid synthesis in the seedlings has already passed, the process can hardly be influenced anymore by mJA. Moreover, in *C. roseus* cell suspension cultures it has already been shown that the other major growth regulators known to regulate alkaloid biosynthesis, auxin and cytokinins, were also active only during the first five days of the subculture (Decendit et al. 1993, Arvy et al. 1994). All these data showed that cell sensitivity to auxin, cytokinin and jasmonates, which leads to a determination of a biochemical differentiation, was restricted to the active cell growth phase. In contrast, the achievement of cell biochemical differentiation, alkaloid synthesis and accumulation, occurred only during the stationary phase.

Jasmonates have been involved as intermediate messengers of wounding, elicitation signals or UV irradiations (Gundlach et al. 1992, Pena-Cortes et al. 1993, Conconi et al. 1996). We have investigated if there was a relationship between the auxin depletion signal, octadecanoic pathway activity and alkaloid biosynthesis. P-cells treated with different octadecanoic pathway inhibitors have a strong reduction in their ability to produce alkaloids. The alkaloid accumulation was always restored by exogenous mJA treatment at a similar level to that observed for p-cells. These results suggested that a functional octadecanoic pathway was necessary for the activation of alkaloid synthesis in response to the auxin depletion signal. The octadecanoic pathway inhibitors acted on jasmonate biosynthesis, but at least part of their action remains unknown. It has been shown that salicylic acid can inhibit tomato proteinase inhibitors (jasmonate induced proteins), by inhibiting the octadecanoic pathway at the level of the conversion of 13S-hydroperoxy linolenic acid into 12-oxophytodienoic acid. But this inhibitor could also act at the level of an unknown mechanism located between jasmonic acid synthesis and transcriptional activation of the proteinase inhibitor gene (Doares et al. 1995). A pleiotropic effect of octadecanoic pathway inhibitors in plant cells cannot be excluded. For these reasons we have tested several octadecanoic pathway inhibitors with different structures, enzymatic targets and action mechanisms (Fig. 5, Pena-Cortes et al. 1993; Farmer et al. 1994). All of them inhibited alkaloid synthesis in p-cells and their inhibitory effect can be reversed by exogenous mJA treatment. These data supported the fact that their inhibitory effect on alkaloid synthesis was a specific consequence, at least partially, of their inhibitory effect on the octadecanoic pathway.

This leads us to the hypothesis that auxin depletion induces biochemical differentiation of *C. roseus* cells by a cascade of physiological events which take place during the first part of the subculture, leading to an activation of the octadecanoic pathway and consequently to an endogenous jasmonate synthesis. Alternatively it could be postulated that auxin had a negative effect on octadecanoic pathway regulation. These hypotheses should now be tested by measuring the endogenous content of auxin and jasmonates in cells cultivated in different physiological conditions.

Our data showed that mJA was able to overcome the inhibition of alkaloid biosynthesis by auxin and to enhance, in an auxin-depleted medium, the production of alkaloids by *C. roseus* cell suspension cultures. The molecular mechanism of the action of mJA on alkaloid biosynthesis should now be investigated.

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