Two distinct intracellular Ca\(^{2+}\)-release components act in opposite ways in the regulation of the auxin-dependent MIA biosynthesis in \textit{Catharanthus roseus} cells

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Abstract

Calcium-mediated signalling is ubiquitous in both animals and plants. Changes in cytoplasmic free Ca\(^{2+}\) concentration couple diverse arrays of stimuli to their specific responses, the specificity of the stimulus being determined by integrated actions between multiple Ca\(^{2+}\) mobilization pathways. In this work, a pharmacological approach is reported, aimed at deciphering the role of calcium as a second messenger in the transduction pathway leading to the inhibitory effect of 2,4-dichlorophenoxyacetic acid (2,4-D), in regulating monoterpene indole alkaloid (MIA) biosynthesis in \textit{Catharanthus roseus} cells. It is demonstrated here that auxin-dependent MIA biosynthesis is differentially regulated by two distinct calcium release components from internal stores in \textit{C. roseus} showing pharmacological profiles similar to those displayed by animal RyR and IP3 channels. MIA biosynthesis is stimulated by caffeine (Ca\(^{2+}\)-release activator through RyR channels) and by heparin and TMB8 (Ca\(^{2+}\)-release inhibitors of IP3 channels) whereas MIA biosynthesis is inhibited by mastoparan (Ca\(^{2+}\)-release activator of IP3 channels) and by ruthenium red and DHBP (Ca\(^{2+}\)-release inhibitors of RyR channels). Furthermore, calcium, as 2,4-D, acts on MIA biosynthesis by regulating the monoterpene moiety of the MIA biosynthesis pathway since calcium channel modulators preferentially modulate \textit{g10h} expression, the gene encoding the enzyme of the secoiridoid monoterpene pathway, that is the major target of 2,4-D action. In addition, the simultaneous use of caffeine (an activator of RyR channel in animals) and TMB8 (an inhibitor of the IP3 channel) in 2,4-D treated cells triggers a synergistic effect on MIA accumulation. This finding suggests an opposite and co-ordinated action of multiple Ca\(^{2+}\)-release pathways in 2,4-D signal transduction, adding a new level of complexity to calcium signalling in plants and questioning the existence of RyR and IP3 channels in plants.

Key words: 2,4-D, calcium signalling, \textit{Catharanthus roseus}, monoterpenoid indole alkaloid.

Introduction

\textit{Catharanthus roseus} (L.) G. Don (\textit{C. roseus}) belongs to the Apocynaceae family. This plant accumulates a diverse spectrum of monoterpene indole alkaloids (MIAs), and some of them are of high pharmaceutical value (Leveque \textit{et al}., 1996). \textit{C. roseus} synthesizes two classes of MIAs: monomeric MIAs, such as ajmalicine, which has anti-hypertensive activity, and dimeric MIAs with anti-neoplastic activity, such as vinblastine and vincristine, which are already used in clinical practice under the trade names of Velban\textsuperscript{®} and Oncovin\textsuperscript{®}. All monomeric and dimeric MIAs are derived from strictosidine, which is formed by the condensation of tryptamine from the shikimate pathway and secologamin from the monoterpenoid secoiridoid biosynthetic pathway (Fig. 1). The regulation of MIA biosynthesis in the plant operates at several levels of complexity. Three pathways (see Fig. 1) are involved in alkaloid synthesis and the genes

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encoding the corresponding enzymes are expressed in various plant tissues (Mahroug et al., 2007).

The levels of biosynthesis and extraction yields for dimeric MIAs are so low in planta, i.e. 0.5 ppm and 5 ppm for vincristine and vinblastine, respectively (Noble, 1990), that cultures of \textit{C. roseus} cells have been developed to increase alkaloid production. The \textit{C. roseus} C20D strain used for alkaloid production requires 2,4-dichlorophenoxy-acetic acid (2,4-D) in the medium for growth (Arvy et al., 1994). The 2,4-D regulates plant growth and development, mimicking naturally occurring auxins, such as indole-3-acetic acid, but it has also been shown that 2,4-D is a powerful inhibitor of alkaloid accumulation in \textit{C. roseus} cells. However, these cells can accumulate MIAs if they are cultured, for at least one cycle, in 2,4-D-free medium (Méillon et al., 1986; Arvy et al., 1994) although only monomeric MIAs accumulate in the absence of cell differentiation. Furthermore, in C20D cells, 2,4-D inhibits the expression of genes encoding enzymes involved in the MEP pathway, i.e. \textit{dxs}, \textit{dxr}, \textit{mecs}, and \textit{hds} genes (Veau et al., 2000; Courdavault et al., 2005; Oudin et al., 2007), and the \textit{g10h} gene (Papon et al., 2005), the first monoterpenoid pathway gene. By contrast, expression of the \textit{sls} and \textit{str} genes, encoding for enzymes involved in the terminal stages of MIA biosynthesis, is 2,4-D independent (Courdavault et al., 2005). Despite the absence of dimeric MIAs from C20D cells, this strain provides a good simplified model for studying the different levels of regulation of MIA biosynthesis. The data obtained in such studies will prove to be useful for the future development of cellular systems for the production of therapeutic compounds.

Little is known about the 2,4-D-dependent signal transduction mechanisms involved in the regulation of MIA biosynthesis. The focus was on the putative role of cytoplasmic calcium in MIA biosynthesis since changes in free cytoplasmic calcium concentration ([Ca^{2+}]_{cyt}) occur during many physiological processes, in response to abiotic stress, pathogens and elicitors (Knight et al., 1991; Grant et al., 2000), heat shock (Gong et al., 1998), cold shock (Knight et al., 1991), drought (Knight et al., 1997), and osmotic effects (Pauly et al., 2001) and particularly in auxin signalling (Lin et al., 2004; Singla et al., 2006). Furthermore, previous authors have suggested that calcium signalling is involved in both cytokinin-induced (Méillon et al., 1991) and methyl jasmonate-induced (Lee-Parsons and Erturk, 2005) accumulation of MIAs within the \textit{C. roseus} cells, however the mechanisms by which calcium controls MIA biosynthesis still remain unknown.

It is now widely accepted that calcium is a ubiquitous secondary messenger in animal and plant cell responses to various extracellular stimuli. The specificity of calcium signalling is controlled by changes in cytosolic free calcium concentration ([Ca^{2+}]_{cyt}) as a function of specific spatial and temporal calcium signature characteristics (McAinsh and Hetherington, 1998; Ng and McAinsh, 2003).

Calcium signalling components from plant and animal systems are highly similar. In plant cells, Ca^{2+} enters the cytosol through plasma membrane Ca^{2+} channels, such as voltage-dependent Ca^{2+} channels, non-selective cation channels, or endomembrane Ca^{2+} channels including voltage-dependent Ca^{2+} channels, such as SV (Slow Vacuolar) and VVCA channels (Vacuolar Voltage-gated Ca^{2+}), and ligand-gated
channels, such as IP3- (Inositol 1,4,5-triPhosphate), cADPR- (cyclic Adenosine DiPhosphoRiboSate) or NAADP- (Nicotinic Acid Adenine Dinucleotide Phosphate) sensitive Ca\textsuperscript{2+}-release channels (see White, 2000, for a review). With the exception of the SV channel, that is formed by dimerization of TPC1 (Two Pore Channel 1) (Peiter et al., 2005), none of the Ca\textsuperscript{2+} channels involved in the release of calcium from internal stores has yet been molecularly characterized in plants, although some data indicate that ligand-gated channels are responsible for [Ca\textsuperscript{2+}]\textsubscript{cyt} elevation (White, 2000) and are involved in some physiological responses such as stomatal closure (MacRobbie, 2000).

In the present work, a pharmacological approach was used to investigate the putative role of calcium in the 2,4-D signal transduction pathway, leading to the regulation of MIA biosynthesis. For this purpose, the effects of well-known specific modulators of animal RyR and IP3 calcium channels on MIA biosynthesis (MIA accumulation and MIA metabolic pathway gene expression) and on [Ca\textsuperscript{2+}]\textsubscript{cyt} variations through aequorin-based were studied luminometry experiments, using a C. roseus cell line constitutively expressing the apoaequorin gene.

The results clearly demonstrate the involvement of two distinct Ca\textsuperscript{2+}-release systems, acting in opposition to one another in the regulation of the 2,4-D-dependent MIA biosynthesis in C. roseus cells and resembling the RyR and IP3 channels found in animals.

Materials and methods

Plant material

The C. roseus (L.) G. Don C20D cell line was maintained in sterile liquid medium containing 3% sucrose, 4.5 μM 2,4-D, and Gamborg’s B5 base (Gamborg et al., 1968) and was subcultured into fresh medium every 7 d. Cultures were grown on shakers (100 rpm), at 25 °C, in the dark.

For bioluminescence experiments C20D cells were transformed by the biolistic method as previously described by Montiel et al. (2007). For this study, cells were bombarded with a mixture of PGL2 plasmids containing a hygromycin selection gene and pIC20H-Aeq plasmids (Knight et al., 1991) containing the apoaequorin gene under the control of the 35S promoter.

One clone (C20D-35S:AEQ) was selected for apoaequorin expression and ajmalicine production. The C20D-35:AEQ cell line was cultured as described above. All the experiments in this study were carried out with the apoaequorin gene-expressing cell line. Similar results were obtained with the wild-type cell line (C20D).

Cells were frozen in liquid nitrogen when used for the preparation of total RNA or freeze-dried when used for the determination of dry weight and ajmalicine accumulation.

Chemicals

All chemicals were purchased from Sigma (St Louis, MO, USA), with the exception of heparin (Calbiochem, Darmstadt, Germany), DHBP (1,1’-DiHeptyl-4,4’-BiPyridinium dibromide; Tocris, Ballwin, MO, USA), mastoparan (Bachem, Bubendorf, Switzerland), and colenterazine (Biosynth AG, Gstaad, Switzerland).

RNA extraction and Northern blotting

Total RNA was extracted from C20D-35:AEQ cells frozen in liquid nitrogen using the RNasey Plant Mini extraction kit (Qiagen, Courtaboeuf, France). For Northern blots, 15 μg of total RNA were fractionated on a 1.0% (w/v) agarose gel containing 2.2 M formaldehyde according to standard methods (Sambrook et al., 1989) and blotted onto a positively charged nylon membrane (Qiogene, Illkirch, France). Probes were prepared by labelling a full-length cDNA with 32P, using the ‘Prime-a-gene’ labelling kit (Promega, Madison, WI, USA). Hybridizations were carried out for 16 h at 60 °C in Church buffer [0.5 M NaPO4 pH 7.2, 7% SDS (w/v), 1 mM EDTA, 1% BSA (w/v)]

Measurement of changes in free cytosolic calcium concentration

Cytoplasmic aequorin was reconstituted in vivo by adding an appropriate volume of C20D-Aeq cells on the seventh day of culture with 2.5 μM colenterazine H and incubating at 25 °C in the dark, with shaking, for at least 2 h. Reconstituted cells (20 μl) were transferred to 96-well microplates and subcultured by adding 180 μl of 2,4-D-free medium 20 min before measurement. Aequorin light emission was recorded for 0.4 s every 3.5 s for each set of conditions, using a microplate luminometer (Mithras LB940, Berthold, Bad Wildbad, Germany). At the end of the experiment, the remaining reconstituted aequorin was eliminated by adding 25 μl of 100 mM CaCl2, 10% (v/v) ethanol, and 2% Nonidet P-40 (v/v). The emitted luminescence, expressed in RLU (relative luminescence units), was used to determine free cytoplasmic Ca\textsuperscript{2+} concentration from a calibration curve (Moyen et al., 1998):

\[
pCa = -\log\left(\frac{[Ca^{2+}]_{cyt}}{k}\right) + 5.593
\]

where k is the rate constant equal to luminescence at any time divided by the sum of the remaining luminescence from this point to the end of the experiment.
Results

Effects of calcium channel modulators on MIA biosynthesis in C20D-35S:AEQ

No significant effects have been observed on MIA biosynthesis in C20D-35S:AEQ, treated with or without 2,4-D, in response to plasma membrane calcium channel blockers (lanthanum chloride, nifedipine, verapamil), extracellular Ca$^{2+}$ chelators (EGTA) or the use of calcium-free medium for cell culture (data not shown). So it was concluded that 2,4-D action on MIA biosynthesis is independent of extracellular calcium availability. Therefore a pharmacological approach was developed to study the putative involvement of endomembrane calcium channels on MIA biosynthesis regulation. Due to the pharmacological similarity between animal IP3 and RyR calcium channels and putative plant IP3- and cADPR-sensitive Ca$^{2+}$ channels, IP3 and RyR specific effectors (agonists and antagonists) were used and their ability to modulate MIA biosynthesis was tested.

Firstly, caffeine, as a known animal RyR’s stimulator (MacLennan et al., 1990), and ruthenium red and DHBP as inhibitors (Kang et al., 1994; Percival et al., 1994) were used. Secondly, mastoparan as a known animal IP3R’s stimulator was used. This molecule mimics an activated G protein-coupled receptor responsible for the activation of phospholipase C (PLC), leading to the production of IP3 from phosphatidylinositol bisphosphate (Komatsu et al., 1992; Legendre et al., 1993). Heparin and TMB8 (8-(N,N-diethylamino)octyl 3,4,5-trimethoxybenzoate) were used as IP3R inhibitors (Nilsson et al., 1988; Förster, 1990).

It has to be noted that caffeine, ruthenium red, mastoparan, heparin, and TMB8 have already been used in plants to modulate [Ca$^{2+}$]_cyt (Bourbouloux et al., 1992; Subbaiah et al., 1994a, b; Franklin-Tong et al., 1996; Cessna et al., 1998; Sun et al., 2007).

Effects of calcium channel modulators on MIA accumulation

The effects of various levels of each effector on ajmalicine accumulation in C20D-35S-35S:AEQ cells were first assessed by adding the compound at the beginning of a cycle of cell culture in 2,4-D-free medium (Fig. 2). Each of the compounds tested had a dose-dependent effect on secondary metabolism (Fig. 2A). Each of these compounds exerted their maximal effects (inhibition or activation) without affecting primary metabolism, as shown by the assessments of cell growth based on dry weight (Fig. 2B).

Caffeine induced very high levels of ajmalicine accumulation (225±3.1% for 500 μM), whereas mastoparan (6 μM) inhibited ajmalicine accumulation (–57±0.1%). Inhibitors of Ca$^{2+}$-release such as ruthenium red and DHBP strongly decreased ajmalicine accumulation (–96±2.25% and –97±0.89% for 5 μM ruthenium red and 10 μM DHBP, respectively), whereas TMB8 (10 μM) and heparin (2 μg ml$^{-1}$) resulted in a small non-significant increase in ajmalicine accumulation.

Effects of calcium channel modulators on MIA gene expression

In C20D cells, MIA accumulation in the absence of 2,4-D is correlated with the expression of genes encoding enzymes of the MIA biosynthetic pathway and especially genes of the monoterpenoid moiety (Papon et al., 2005). The role of calcium in MIA biosynthesis was studied in more detail by carrying out a Northern blot analysis of genes encoding

\[ \text{Fig. 2. Effects of Ca}^{2+} \text{ release modulators on ajmalicine accumulation (A) and on cell growth (B). } \]

C. roseus C20D-35S:AEQ cell lines were subcultured in a 2,4-D-free medium (without 2,4-D), in the presence of different levels of calcium channel effectors added at the initiation of the cell culture cycle. Cells were harvested after 10 d of culture for ajmalicine quantification and cell growth measurements. (A) Results are expressed as variation (%) between treated and control cells ± standard error (n=3) * P <0.05; ** P <0.005; *** P <0.0005. (B) Results representing the dry weight of cells for a flask containing 50 ml of cell culture.
three specific enzymes in this branch of MIA biosynthesis pathway.

Firstly, Northern blot analysis was carried out for the \textit{g10h}, \textit{sls}, and \textit{str} genes (see Fig. 1), in C20D-35S:AEQ cells, over a complete cell culture cycle. These genes were chosen because of the crucial position of the enzymes they encode in the MIA biosynthesis pathway and because of their sensitivity to auxin. G10H and SLS are the first and last enzymes, respectively, in the monoterpenoid secoiridoid pathway and STR is the first enzyme of the terminal branch of the MIA biosynthesis pathway (Fig. 1). Furthermore, 2,4-D is known to have a selective effect on the first steps of the monoterpenoid branch of the MIA biosynthesis pathway (Arvy et al., 1994; Oudin et al., 2007).

In the presence of 2,4-D, \textit{g10h} transcripts were detectable after the fifth day and reached maximal levels on the sixth day, although expression levels remained low. Under the same conditions, \textit{sls} and \textit{str} were constitutively expressed with expression levels peaking between the third and fourth days of culture (Fig. 3). In 2,4-D-free medium (MIA production medium), \textit{g10h} transcripts were detected two days earlier than in the presence of 2,4-D, i.e. on the third day of culture. In complete accordance with the beginning of ajmalicine accumulation in C20D cells, the levels of \textit{g10h} expression increased over time, peaking on the sixth day of culture at a higher level than the one observed in the presence of 2,4-D. \textit{Sl} and \textit{str} displayed constitutive expression throughout the entire cell culture cycle. In both conditions, the low level of \textit{g10h} transcripts detectable on day 0 corresponded to residual transcripts present on the last day (day 7) of the previous cell culture cycle. These gene expression kinetics provide a fingerprint of the C20D-35S:AEQ cell line, making it possible to establish experimental conditions for testing the effect of calcium channel effectors on the expression of these three specific genes. Our observations also strongly support previous findings of an effect of 2,4-D on \textit{g10h} expression, as reported for the C20D cell line (Papon et al., 2005).

Secondly, the role of endomembrane calcium channel modulation in the MIA biosynthesis pathway was investigated by carrying out Northern blotting for the \textit{g10h}, \textit{sls}, and \textit{str} genes in C20D-35S:AEQ cells cultured in 2,4-D-free medium and treated with caffeine, mastoparan, ruthenium red, DHBP, heparin or TMB8. Based on the effects of calcium channel effectors on MIA accumulation (Fig. 2) and our analysis of \textit{g10h} expression over an entire culture cycle (Fig. 3), \textit{g10h}, \textit{sls}, and \textit{str} expression was analysed on day 6 for inhibitors of MIA accumulation and on day 5 for activators of MIA accumulation.

Caffeine and heparin were both shown to lead to an increase in \textit{g10h} transcript levels. Mastoparan, ruthenium red, and DHBP were all shown to inhibit \textit{g10h} expression (Fig. 4). TMB8 had no significant effect on \textit{g10h} transcript levels. Mastoparan concomitantly led to an increase in both \textit{sls} and \textit{str} transcript levels and ruthenium red weakly inhibited \textit{str} expression. The expression patterns of \textit{sls} and \textit{str} were not modified by caffeine, DHBP, heparin or TMB8.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig3}
\caption{RNA gel blot analysis of \textit{g10h}, \textit{sls}, and \textit{str} transcript levels during a cell cycle. Total RNA was extracted from a \textit{C. roseus} C20D-35S:AEQ cell line at various days after subculture in 4.5 \(\mu\)M 2,4-D medium (+2,4-D) or in a 2,4-D-free medium (-2,4-D) and subjected to Northern blot analysis using \textit{g10h}, \textit{sls}, and \textit{str} cDNA \(^{32}\text{P}\)-labelled probes. Equal loading of RNA for each sample was confirmed by ethidium bromide (EtBr) staining. Each result is representative of three independent Northern blot analyses. The numbers indicates the delay between subculture and harvest. Day 0 was harvest 30 min after subculture.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig4}
\caption{RNA gel blot analysis of \textit{g10h}, \textit{sls}, and \textit{str} transcript levels in calcium channels effectors-treated \textit{C. roseus} C20D-35S:AEQ cells. A \textit{C. roseus} C20D-35S:AEQ cell line was treated with increasing doses of \(\text{Ca}^{2+}\) release activators (caffeine and mastoparan) and \(\text{Ca}^{2+}\) release inhibitors (ruthenium red, DHBP, heparin, and TMB8). According to Fig. 2, total RNA was extracted on the sixth day of culture for effectors inducing inhibition of ajmalicine accumulation and on the fifth day of culture for effectors inducing activation of ajmalicine accumulation. Samples were subjected to Northern blot analysis using \textit{g10h}, \textit{sls}, and \textit{str} cDNA \(^{32}\text{P}\)-labelled probes. All effectors concentrations are given in \(\mu\)M except for heparin where quantities are given in \(\mu\)g ml\(^{-1}\). Equal loading of RNA samples was confirmed with ethidium bromide (EtBr) staining. Each result is representative of three independent Northern blot analyses.}
\end{figure}
It should be mentioned that the concentrations of all the drugs used here were similar to those commonly used in animal models and were much lower than those generally used in plant models (West and Williams, 2007).

Comparing these results with those already reported for the specificity of the drugs in animal models, it can be assumed that RyR’s agonists induce MIA biosynthesis whereas RyR’s antagonists inhibit MIA biosynthesis. Opposite results were observed for the IP3R’s modulators, mainly with the IP3R’s stimulator which inhibits MIA biosynthesis. These results (MIA accumulation and gene expression) suggest that two distinct intracellular Ca2+-release components are involved in the regulation of MIA biosynthesis. The first component is activated by caffeine and inhibited by ruthenium red and DHBP and acts in a positive way on MIA biosynthesis, whereas the second component is activated by mastoparan and inhibited by TMB8, and more specifically by heparin, and acts to repress MIA biosynthesis. Based on the pharmacological properties of these effectors, already reported on animal models but also on plant models, it can be hypothesized that the putative cADPR-sensitive Ca2+-release channel acts positively whereas the putative IP3-sensitive Ca2+-release channel acts negatively on MIA biosynthesis.

In order to confirm that the various pharmacological agents affecting MIA biosynthesis and accumulation proceed through [Ca2+]cyt variations, aequorin-based luminometry experiments were performed to determine [Ca2+]cyt in response to these drugs.

**Ca2+ release in C. roseus cells in response to animal RyR and IP3 channel modulators**

The ability of caffeine and mastoparan to increase free cytosolic Ca2+ concentration was assessed. The C20D-35S:AEQ cell line was incubated with coelenterazine to reconstitute a functional aequorin for calcium measurements. C20D-35S:AEQ cells were then subcultured in 2,4-D-free medium for 20 min and caffeine or mastoparan was added to the cell suspension without mixing to prevent any mechanical disturbances of the cells which would probably elicit strong [Ca2+]cyt transients (data not shown).

Mastoparan induced a dose-dependent increase in [Ca2+]cyt, which peaked at 1.17±0.04 µM 30–50 s after treatment (Fig. 5A) for a mastoparan concentration of 1392 | Poutrain et al.
IP3-sensitive Ca²⁺-release channel, using the IP3R inhibitor to confirm the auxin-mediated regulation of the monoterpene biosynthesis pathway. An attempt was made to confirm the auxin-mediated regulation of the monoterpene accumulation (1720 ± 500 μM). Caffeine, at a concentration of 500 μM—the optimal concentration required for a maximal effect on ajmalicine accumulation with no effect on cell growth—induced a small increase in [Ca²⁺]cyt (Fig. 5C). A dose-dependent increase in [Ca²⁺]cyt was observed while increasing the caffeine concentration. A peak of 307 ± 20 nM is reached at a concentration of 1.5 mM caffeine. The increase in [Ca²⁺]cyt induced by 1 mM caffeine was inhibited by simultaneously adding ruthenium red and caffeine (Fig. 5D). This inhibition was dose-dependent going from 60% to full inhibition using 12.5 μM and 25 μM ruthenium red, respectively. Thus, the compounds studied here regulate [Ca²⁺]cyt by modulating Ca²⁺-release channels activities in C. roseus.

In order to study the putative involvement of endomembrane calcium channels in the transduction of the 2,4-D signal leading to an inhibition of MIA biosynthesis, the ability of endomembrane calcium channel effectors to reverse the inhibitory effects of 2,4-D, like caffeine and TMB8 that are known to stimulate MIA accumulation, was assessed.

Reversion of the inhibitory effect of 2,4-D on MIA biosynthesis by calcium channel modulators

C. roseus cells were cultured in medium containing 1.5 μM 2,4-D. At this low concentration of 2,4-D, cells were able to accumulate small amounts of MIAs, whereas no MIAs were detected if cells were cultured in classical 2,4-D-medium (4.5 μM). These experimental conditions were used to reveal the maximum effect of calcium channel modulators on MIA accumulation in the presence of 2,4-D.

The effects of TMB8 and caffeine, applied separately or together, on MIA accumulation (Fig. 6) were assessed. With 2,4-D present in the medium, 500 μM caffeine and 10 μM TMB8 greatly increased ajmalicine accumulation (580±160% and 83%±50%, respectively). The activation of MIA accumulation by caffeine and TMB8 was stronger in the presence of 1.5 μM 2,4-D than with no auxin in the medium (Fig. 2), where activation rates were 225% for 500 μM caffeine and 33% for 10 μM TMB8. Interestingly, the addition of TMB8 and caffeine together resulted in a synergistic, rather than an additive effect on MIA accumulation (1720±760%).

The results presented in Figs 3 and 4 strongly suggest that calcium, as well as 2,4-D, affect a specific branch of the MIA biosynthesis pathway. An attempt was made to confirm the auxin-mediated regulation of the monoterpene branch of the MIA biosynthetic pathway by studying the effects of various ajmalicine precursors of this pathway in the presence of ruthenium red.

Ruthenium red, like 2,4-D, preferentially inhibits the terpenoid branch of MIA biosynthesis

It has been shown that the addition of secoiridoid monoterpene precursors, in the presence of 2,4-D, restores the ability of C20D cells to accumulate ajmalicine (Arvy et al., 1994). These findings confirm that the effect of 2,4-D on MIA biosynthesis primarily concerns the secoiridoid monoterpene pathway. In this study, it was found that ruthenium red strongly inhibited MIA accumulation (Fig. 2). Nevertheless, in the presence of this animal RyR antagonist, C. roseus cells supplied with loganic acid (300 μM), loganin (300 μM), and secologanin (300 μM) (Fig. 7) displayed almost normal bioconversion of these terpenoid precursors (15% versus 20% for the control). This suggests that ruthenium red acts on MIA biosynthesis upstream from loganic acid in the terpenoid branch. The weak inhibition of bioconversion in the presence of ruthenium red is consistent with the mild inhibitory effect of ruthenium red on str (downstream from secologanin) gene expression (Fig. 4).

Discussion

The monoterpene indole alkaloid biosynthesis is regulated by two Ca²⁺ release components from internal stores

Involvement of calcium in the regulation of secondary metabolism in higher plants has been shown in several studies. Indeed, calcium deprivation in Silybum marianum cell cultures, or the inhibition of increases in cytosolic calcium concentration by calcium chelators or channel inhibitors, regulate silymarin accumulation (Sanchez-Sampedro

![Fig. 6. Effects of caffeine and TMB8 on ajmalicine accumulation in 2,4-D treated cells. C. roseus C20D cell line were subcultured in medium supplemented with 1.5 μM of 2,4-D. Caffeine (500 μM) or/ and TMB8 (10 μM) were added at the initiation of the culture cycle. Cells were harvested after 10 d for ajmalicine quantification. Results are expressed as a variation (%) between treated cells and control cells for the same experiments. The standard error is shown.](image-url)
Fig. 7. Effects of ruthenium red, a RyR inhibitor, on terpenoid bioconversion into ajmalicine in C20D-35S:AEQ cells. C. roseus C20D-35S:AEQ cell lines were subcultured in 2,4-D-free medium with ruthenium red (5 μM) (RR) or with water (control) added at the initiation of the culture cycle. Loganic acid, Loganin or Secologanin (all at 300 μM) were added at the start of the third day of the culture cycle. Cells were harvested 10 d post-treatment for ajmalicine quantification. Results are presented as the rate of each terpenoid precursor converted into ajmalicine and are representative of three independent experiments. The standard error is shown.

et al., 2005). Ca^{2+} influx into the cytosol is also required for elicitor-induced production of the phytoalexin β-thujaplicin in Cupressus lusitanica cell cultures (Zhao and Sakai, 2003), and for elicitor-induced phytoalexin biosynthesis in soybean (Glycine max) (Stab and Ebel, 1987), bloodroot (Sanguinaria canadensis) suspension cultures (Mahady and Beecher, 1994), and oat leaves (Ishihara et al., 1996). Increasing extracellular Ca^{2+}, as well as the use of the calcium ionophore A23187, also stimulate the production of tropane alkaloids in cell suspensions of Datura innoxia (Gontier et al., 1994) and hairy root cultures of Brugmansia candida (Pitta-Alvarez et al., 2000).

In the case of C. roseus cells, previous works have suggested the involvement of calcium in the regulation of monoterpeneoid alkaloid accumulation. Indeed, calcium influx into the cytosol and the action of calcium-calmodulin are required for cytokinin-induced alkaloid accumulation in the C20D and CR6 cell lines (Mérimon et al., 1991). Methyl jasmonate-stimulated alkaloid accumulation in A11 cell suspensions is also dependent on the influx of extracellular calcium (Lee-Parsons and Erturk, 2005). By contrast, the use of TM88 or thapsigargin, a drug responsible for the depletion of intracellular Ca^{2+} stores, increases alkaloid accumulation in the LM2 hairy root line (Moreno-Valenzuela et al., 2003).

In this study, our work focused on the role of intracellular calcium release since 2,4-D action on MIA biosynthesis seems to be independent of the calcium influx from the external stores. The possible involvement of two endomembrane calcium channels in the regulation of MIA biosynthesis in C. roseus cells was investigated. Using several well-known animal calcium channel effectors, it was shown that the accumulation and biosynthesis of MIAs could be regulated by two Ca^{2+}-release components involving ligand-gated channels (Figs 2, 4). Furthermore, it was shown that two compounds (caffeine and mastoparan), known to mobilize Ca^{2+} from internal stores, trigger opposite responses on ajmalicine accumulation (Fig. 2) and on gene expression (Fig. 4). Moreover, it was shown that these compounds are able to induce [Ca^{2+}]_cyt variations in C. roseus cells (Fig. 5). Finally, specific inhibitors of animal RyR channels, such as ruthenium red and DHBP, or specific inhibitors of animal IP3 channel, such as heparin and TM88, trigger opposite effects on MIA biosynthesis.

The specificity of the pharmacological compounds, used to modulate calcium homeostasis in plants, can indeed be questioned, but it has been shown that caffeine and ryanodine, two compounds known to activate animal RyR channels, are able to induce [Ca^{2+}]_cyt transiently in plant cells (Subbiah et al., 1994a, b) and it has been shown that ruthenium red, a RyR specific inhibitor, also inhibits cADPR-sensitive Ca^{2+}-release in plants (Cessna et al., 1998). Nevertheless, some data indicate that, in plant cells, ruthenium red also acts to inhibit the rca channel, the maxication and the SV channels, suggesting a lack of specificity for this compound (White, 2000).

Even if the specificity of ruthenium red is questionable, it is shown that this compound inhibits caffeine-induced Ca^{2+}-release (Fig. 5) and also inhibits g10h gene expression and ajmalicine accumulation as previously observed with DHBP, another inhibitor of animal RyRs (Kang et al., 1994). These results suggest that these two compounds act on MIA biosynthesis through a modulation of cADPR-sensitive Ca^{2+}-release. Furthermore, other RyR inhibitors, such as procaine (Yue et al., 1995) and ryanodine, were also tested for their ability to modulate MIA accumulation. Procaine inhibits ajmalicine accumulation in C20D and C20D-35S:AEQ cell lines and ryanodine, at a low level (<0.5 μM), activates MIA accumulation whereas at a high level (>0.5 μM), ryanodine inhibits MIA accumulation (data not shown). In conclusion, these data suggest that the animal-specific RyR compounds used in this study, also act on the plant calcium signalling component resembling that of the animal RyR channel.

Furthermore, mastoparan, a peptide mimicking G protein activated receptor is known to activate calcium release via the PLC/IP3 pathway in both animal and plant cells (Tucker and Boss, 1996) but it has been also suggested that mastoparan could induce calcium releases without requiring G proteins (Sun et al., 2007). Furthermore, several studies on both plant and animal models show that mastoparan could permeabilize the plasmalemma and induce calcium influx (van Himbergen et al., 1999; Jones and Howl, 2006). However, it appears that this phenomenon occurs for higher levels of the peptide (>20 μM) than those used in our model (<6 μM). Furthermore, it was shown that an associated form of PLC, purified from C. roseus roots, could interact with heparin (Echevarría-Machado et al., 2007). Here, it is clearly shown that heparin inhibits mastoparan-induced Ca^{2+}-release (Fig. 5B) indicating a possible mastoparan-activated PLC activity in C. roseus cells. Lithium chloride was also tested on MIA biosynthesis under the same condition.
conditions as reported here. LiCl is a known inhibitor of inositol-1-phosphate phosphatase, which results in a reduction in inositol cycling (Honchar et al., 1989) and in a reduction of IP3 synthesis (Ortega and Pérez, 2001). In the presence of 1 μM LiCl, a 20% (±7%) increase was observed in ajmalicine accumulation (data not shown). Altogether these data strengthen the hypothesis of IP3 involvement.

Based on similar pharmacological profiles between animals and plants and despite any clear molecular evidence for the existence of IP3 or cAPDR receptors in plants, our results tend to demonstrate that these two Ca2+-release mechanisms are involved in the regulation of MIA biosynthesis in *C. roseus* cells and may be linked to IP3- and cADPR-sensitive Ca2+-release channels.

The existence and localization of these two channels in plant cells remain to date, open questions. Since no genes coding the proteins have yet to be identified in any available plant genome, only pharmacological studies indicate endomembranar localization of putative channels responding to IP3 and cADPR. Studies on microsomes from *Chenopodium album* (Lommel and Felle, 1997) and *B. vulgaris* (Allen et al., 1995) indicated that cADPR- and IP3-induced Ca2+ release have a vacuolar origin. Indeed, patch clamp experiments, performed on isolated tonoplasts from *B. vulgaris*, have shown the current modification at negative potentials in the presence of IP3 (Allen and Sanders, 1994) or cADPR (Allen et al., 1995) but it appears that the vacuolar activities of the two channels is negligible in comparison with the activities from non-vacuolar stores (Muir and Sanders, 1997). Furthermore, in cauliflower, the origin of cADPR-mobilized calcium release seems to come from the rough endoplasmic vesicles (Navazio et al., 2001) and, in *C. rubrum*, high affinity IP3 binding sites were found in the endoplasmic reticulum fraction (Martinec et al., 2000).

Based on our results, it is hypothesized that these two Ca2+-release mechanisms are carried out by the IP3- and cADPR-sensitive Ca2+-release channels which act on MIA biosynthesis in an opposite manner.

Is calcium signalling implicated in the transduction of the 2,4-D signal, regulator of MIA biosynthesis?

Many reports have suggested that calcium acts as a secondary messenger in auxin signalling. 2,4-D induces calcium influx in the cytosol of parsley hypocotyls and corn coleoptiles (Gehring et al., 1990) and it was shown that 1-NAA induces a rapid increase of [Ca2+]cyt in wheat protoplasts (Shishova and Lindberg, 2004) via the mobilization of extracellular calcium, but also of vacuolar calcium. Furthermore, 2,4-D induces Ku70 and Ku80 gene expression. Ku70 and Ku80 are involved in the DNA repair process and the maintenance of telomeres through mechanisms implicating calcium influx and calmodulin (Liu et al., 2007). It was also shown that calcium and TCH3, a CaM-related protein, are involved in PID kinase activity (Benjamin et al., 2003).

In *C. roseus* cells, 2,4-D is a powerful inhibitor of MIA biosynthesis as it inhibits both MIA accumulation (Mériillon et al., 1986) and expression of genes encoding the monoterpene moiety of the biosynthetic pathway (Oudin et al., 2007). In the current study, it is shown that caffeine (activator) and TMB8 (inhibitor) are able to reverse the 2,4-D inhibitory effect on MIA accumulation, suggesting an involvement of Ca2+ signalling in the 2,4-D signal transduction governing MIA biosynthesis. In addition, the simultaneous use of the two chemicals results in a synergistic response on ajmalicine accumulation, confirming the involvement of two distinct Ca2+-release components and suggesting a combined action of these components on MIA biosynthesis regulation.

It is hypothesized that 2,4-D inhibits cADPR-dependent calcium release and/or activates IP3-dependent calcium release. One possible explanation for this 2,4-D action could be CICR (Calcium-Induced Calcium Release) where calcium released from the RyR channel activates the IP3 channel (or a similar plant calcium-release component to the animal RyR and IP3 channels) having an inhibitory effect on alkaloids production, thus lowering the stimulatory effect of calcium released through RyR. Once the IP3 receptor is inhibited with TMB8, then the stimulatory effect, due to calcium released from RyR, can be fully expressed. So, if this phenomenon is currently appearing, it is impossible to measure the calcium specifically released through the IP3 receptor under CICR because cytosolic aequorin is measuring all the cytosolic calcium whatever the sources are.

The Ca2+ channels modulators, used in this study, mainly target expression of the *gloh* gene (Fig. 4), a crucial gene encoding the first enzyme of the secoiridoid monoterpene pathway, as observed for 2,4-D (Fig. 3) (Papon et al., 2005). The expression of *sls* and *str* was 2,4-D-independent (Courdavault et al., 2005) and was unaffected by most of the drugs compounds tested here.

It has also been shown that supplying C20D cells, cultured in 2,4-D medium, with terpenoid precursors such as loganic acid, loganin, and secologanin, can restore the ability of the cells to accumulate ajmalicine (Arvy et al., 1994). It is shown here that, even in the presence of ruthenium red, C20D-3SS:AEQ cells can convert terpenoid precursors into ajmalicine (Fig. 7). This suggests that calcium mobilized from internal stores regulates MIA biosynthesis upstream from loganic acid in the same manner as 2,4-D.

Taken together, these results demonstrate that the regulatory effects of calcium ions on MIA biosynthesis mostly concern the first half of the secoiridoid monoterpene pathway, specially the G10H step. These regulatory effects are exerted at least at a transcriptional level, like those of 2,4-D. Thus, calcium may play a key role in the 2,4-D-dependent MIA biosynthesis in *C. roseus* cells since it has also been shown, in aequorin-cells line, that 2,4-D induced a rapid and transient [Ca2+]cyt increase, in a dose-dependent manner, peaking 5 min after treatment (O Pichon, personal communication).
Putative interaction between IP3- and cADPR-sensitive Ca\(^{2+}\)-release channels in the regulation of 2,4-D-dependent MIA biosynthesis

Studies on vacuolar K\(^+\) release in stomatal guard cells provide useful data to understand possible interaction between the IP3 and cADPR pathways. Experiments with photolysis of caged-IP3 as well as cADPR microinjection produce stomatal closure (Blatt et al., 1990; Leckie et al., 1998) and IP3 and cADPR levels are increased upon ABA treatment (Lee et al., 1996; Wu et al., 1997). Furthermore, MacRobbie (2000) shows that ABA-induced release of vacuolar ions, in \textit{C. communis}, is dependent on an increase in [Ca\(^{2+}\)]\textit{cyt} arising from both an influx of Ca\(^{2+}\) from extracellular pools and its release from internal stores via PLC- and cADPR-dependent processes. These data strongly suggest that both IP3- and cADPR-sensitive Ca\(^{2+}\)-release channels contribute to the ABA-dependent stomatal closure.

In the case of the MIA biosynthesis in \textit{C. roseus} cells, where the cADPR-dependent pathway has a positive effect on MIA biosynthesis whereas the IP3-dependent pathway has a repressive effect, it has been shown that these two pathways have a combined and an interdependent action for governing the 2,4-D-dependent MIA biosynthesis, since synergy was observed while modulating the two pathways (Fig. 5).

These data, obtained in a plant model, are consistent with data coming from animal models where a novel approach to understanding the specificity of calcium signalling, for a given physiological process, involves communication between channels (Berridge, 2006a). The cellular response to a given stimulus not only depends on specific changes in calcium concentrations, as in the ‘calcium signature model’, but also on the various channels involved in these changes due to their location in the direct micro-environment. Numerous works have shown the existence of cytoplasmic calcium micro-domains, surrounding the Ca\(^{2+}\) channels, constituting the basic functional features assigning the specificity of response to a given stimulus (Berridge, 2006b).

Ca\(^{2+}\) microdomains are generally defined as any increase in cytoplasmic Ca\(^{2+}\) concentration which is restricted to one part of the cell or forming a local hot spot very close to Ca\(^{2+}\) channels (Berridge, 2006a). For example, in both skeletal and cardiac muscle, RyRs channels are present in specialized portions of the sarcoplasmic reticulum, the terminal cisternae that is facing the t-tubule. This microstructure plays a key role in generating the calcium increase driving muscle contraction (Franzini-Armstrong and Protasi, 1997).

The molecular characterization of plant’s IP3- and cADPR-sensitive Ca\(^{2+}\)-release channels would greatly contribute to confirm the hypothesis for the existence of these two channels and their contribution to the spatial distribution of calcium-dependent signalling components in the specificity of the cellular response in plants. Fluorescence imaging techniques, as well as the characterization of downstream calcium signalling components like calmodulin or calcium-dependent protein kinase, would help complement our knowledge on the hormonal regulation of the secondary metabolism, driven by auxin.

For this purpose, the model of MIA biosynthesis regulation by 2,4-D in \textit{C. roseus} cells, seems to be a good model for studying a putative new level of complexity in calcium signalling in plants, such as the co-ordinated action of Ca\(^{2+}\)-release channels in cellular microstructures.

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