Cytokinin and Ethylene Control Indole Alkaloid Production at the Level of the MEP/Terpenoid Pathway in *Catharanthus roseus* Suspension Cells

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Abstract

The Madagascar periwinkle *Catharanthus roseus* accumulates a number of terpenoid indole alkaloids, some of which have high therapeutic interest. The biotechnological approach with cells *in vitro* remains an alternative to the field culture of periwinkle for the production of such compounds. We previously reported that two phytohormones, cytokinin and ethylene, remarkably enhanced the accumulation of alkaloids in periwinkle cell suspensions. In this work, we investigated the effects of these hormones on the regulation of several genes of the indole alkaloid biosynthetic pathway. We show that cytokinin and/or ethylene greatly enhanced the expression of the geraniol 10-hydroxylase gene. When given together, these hormones also increased the expression of three genes belonging to the methyl-erythritol pathway. These results make it possible to consider elements of cytokinin and ethylene signalling pathways as tools for improving terpenoid indole alkaloid production through metabolic engineering.

Catharanthus roseus (L.) G. Don (Madagascar periwinkle) is a tropical Apocynaceae which accumulates a wide range of terpenoid indole alkaloids (TIAs), including the anti-hypertensive drug ajmalicine and the very efficient anti-cancer drugs vinblastine and vincristine. TIAs are synthesised by more than twenty enzymes in cytosol, (pro)vacuoles, and chloroplasts/plastids [1] (Fig. **1**). Their common precursor is strictosidine, which results from the stereospecific condensation of tryptamine and secologanin by strictosidine synthase (STR). Tryptamine is formed by decarboxylation of l-tryptophan by tryptophan decarboxylase (TDC) while secologanin is derived from the terpenoid pathway coming from isopentenyl diphosphate (IPP). Recently, IPP required for TIA production has been shown to be synthesised mainly through the alternative methyl-erythritol phosphate (MEP) pathway in plastids rather than through the canonical mevalonate pathway in cytosol [2], [3]. Although TIAs used in therapy are still extracted from field-grown plants of *C. roseus*, cell cultures are always receiving considerable interest for investigating treatments/methods that may increase TIA accumulation in suspension cells [1]. Several reports, including precursor feeding experiments, have shown that limiting steps in the biosynthetic pathway of monomeric TIAs in suspension cells occur mainly at the level of the terpenoid branch [4], [5], [6], [7]. In this context, the enzyme geraniol 10-hydroxylase, which catalyses the conversion of geraniol/nerol into their 10-hydroxy derivatives, seems to play a major regulatory role [6], [8], [9], although other key steps may exist [7].

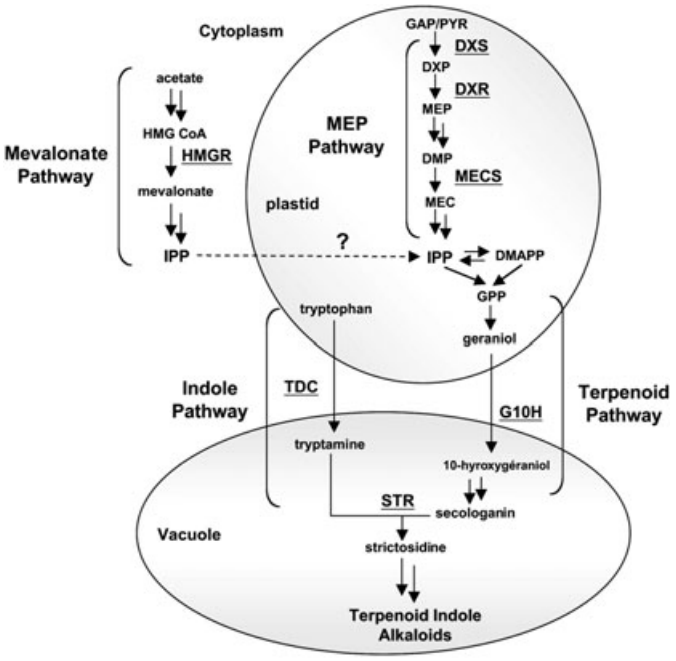

Fig. **1** Synthesis of TIAs in Madagascar periwinkle. Single arrows indicate one-step enzymatic conversion and double arrows indicate multiple-step enzymatic conversions. Biosynthetic genes studied in this work are underlined. IPP: isopentenyl diphosphate; DMAPP: dimethylallyl diphosphate; DMP: 4-diphosphocytidyl-2-C-methyl-D-erythritol 2-phosphate; DXS: 1-deoxy-D-xylulose 5-phosphate (DXP) synthase; DXR: DXP reductoisomerase; MECS: 2C-methyl-D-erythritol 2,4-cyclodiphosphate (MEC) synthase; MEP: methyl-erythritol phosphate; GAP: glyceraldehyde 3-phosphate; GPP: geranyl diphosphate; G10H: geraniol 10-hydroxylase; HMGR: 3-hydroxy-3-methylglutaryl-CoA reductase; PYR: pyruvate; STR: strictosidine synthase; TDC: tryptophan decarboxylase.

We are currently studying the regulation of TIA biosynthesis in *C. roseus* suspension cells. The C20D line we use does not produce any TIAs when grown in its maintenance medium (containing 2,4-dichlorophenoxyacetic acid, 2,4-D); however, in these conditions, the indole branch of the TIA pathway remains functional since adding secologanin (the final metabolite of the terpenoid branch) to the culture medium leads to TIA accumulation in the cells [10]. In fact, deleting 2,4-D from the medium induces both the production of ajmalicine (see Ctr in Fig. **2**) and the expression of 1-deoxy-D-xylulose 5-phosphate synthase (*dxs)* and 1-deoxy-D-xylulose 5-phosphate reductoisomerase *(dxr)* genes [11]; therefore, some limiting steps of TIA biosynthesis exist at the level of the MEP pathway in this line. We also found that adding cytokinin (CK) or ethylene (given through ethefon degradation) at the 4th day of culture increases the TIA accumulation without affecting the cell growth [12]. Co-adding both hormones further

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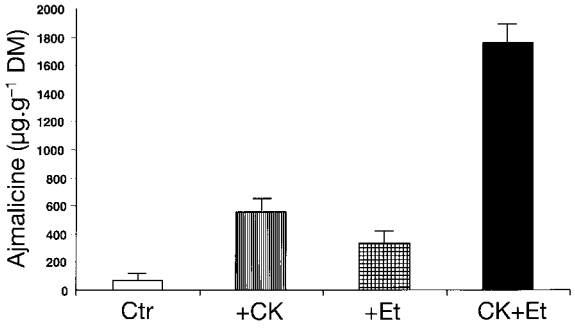

Fig. **2** Cytokinin and/or ethylene enhance the TIA production in C20D cells subcultured in 2,4-D-free medium. Cells were treated on the 4th day of culture with either 5 µM of *trans*-zeatin (CK), 0.1 mM ethefon (Et) or both (CK + Et). Ajmalicine contents were quantified after 72 hours of hormone treatment. Ctr: untreated (control) cells grown without 2,4-D. (Cells grown in the presence of auxin do not accumulate any TIA).

greatly enhances the TIA production (Fig. **2**). The aim of the present work was to investigate which genes of the three branches (i.e., mevalonate, MEP or indole branch) of the TIA biosynthetic pathway are controlled by these hormones.

In a first series of experiments, we examined whether a relationship exists between TIA accumulation and geraniol 10-hydroxylase (*g10h)* gene expression in C20D cells. As reported for another periwinkle line [6], the expression of *g10 h* was undetectable in C20D cells grown in the presence of 2,4-D (Fig. **3**, **A**) but was induced, concomitantly with a slight increase of TIA accumulation in cells grown in 2,4-D-free medium (Fig. **3**, **B**). Addition of CK or ethylene that increased the amounts of ajmalicine, the main monomeric TIA in the cells (Fig. **2**), further enhanced the contents of *g10 h* transcripts within 24 h of treatment (Fig. **3**, **B**). A fully habituated line C20A derived from C20D [10], which does not accumulate any TIA even after treatment with CK and/or ethylene, showed no expression of *g10 h* gene in any conditions (Fig. **3**, **C**).

In a second series of experiments, we compared the expression of *g10 h* with that of other genes of the TIA pathway in cells treated

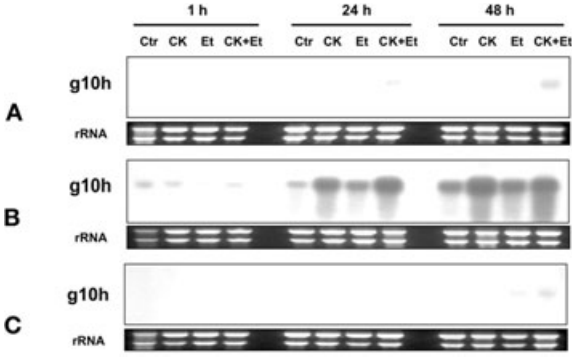

Fig. **3** Effect of cytokinin and/or ethylene on *g10 h* gene expression in C20D cells grown in 2,4-D-containing- (**A**) or 2,4-D-free medium (**B**). The fully habituated C20A line (maintained in 2,4-D-free medium) is shown as reference for non-producing cells (**C**). Cells were treated on the 4th day of culture with 5 µM of *trans*-zeatin (CK), or 0.1 mM ethefon (Et), or both (CK + Et). Ctr = untreated cells. Cells were harvested after 1, 24 or 48 hours of treatment.

with CK and/or ethylene (Fig. **4**). Given alone, the hormones had no or little effect on the expression of genes belonging to either the MEP pathway (*dxs*, *dxr* and *mecs*) or the mevalonate pathway (*hmgr*); they also did not significantly modify the accumulation of *tdc* and *str* transcripts. Given together, the hormones not only strongly enhanced *g10 h* expression but also clearly increased the expression of the MEP pathway genes. Again, no effect was noticed for *tdc*, *hmgr* and *str* gene expressions, which agrees with the fact that tryptamine is not limiting in C20D cells [10] and that the mevalonate branch seems to have a minor role in TIAs synthesis [2].

A first observation is that the enhancement of TIA accumulation in C20D cells treated with CK and/or ethylene is associated with an increase in *g10 h* transcripts. We previously noticed that zeatin enhanced the geraniol 10-hydroxylase activities in C20D cells [13] and other works have shown that the stress phytohormone methyl jasmonate induces *g10 h* expression [9] and enhances TIA accumulation [14] in periwinkle suspension cells. All these results confirm the key regulatory role of geraniol 10-hydroxylase in the TIA pathway, even if other upstream and/or downstream steps may be involved. A question arises as whether the effect of CK in C20D cells is mediated through induction of ethylene production, as reported in several models [15]: this may be ruled out since we found that treating C20D cells with CK led to a decrease in ethylene production [12]. CK and ethylene likely act through distinct mechanisms which converge on genes involved in early steps of the TIA biosynthesis. In this perspective, a cross-talk between the CK and ethylene transduction pathways has been recently proposed at the level of the response regulator ARR2 in *Arabidopsis thaliana* [16]. This may explain the second observation showing another step of regulation at the MEP pathway when CK and ethylene are applied simultaneously. Elements that link cytokinin and ethylene signalling to the expression of *dxs*, *dxr, mecs* and *g10 h* could be powerful tools to manipulate genetically the MEP/terpenoid pathway, in order to obtain TIA high producing periwinkle strains.

Materials and Methods

Plant materials and growth conditions: C20D cells were maintained on a 7-day-growth cycle in 250-mL Erlenmeyer flasks containing each 50 mL medium supplemented with 58 mM sucrose and 4.5 µM 2,4-D [10], [11]. Fully habituated C20A cells [10] were maintained in 2,4-D-free medium. All cell suspensions were shaken (100 rpm) at 25 °C in the dark.

TIA quantitation: Ajmalicine was chosen as a marker of TIA accumulation and quantified by fluorescence densitometry [11]. We have checked that the accumulation pattern of other TIAs (serpentine, catharanthine) parallels that of ajmalicine (data not shown).

RNA gel blot analysis: See [11] for the procedure. The following ^{32}P-labeled full-length cDNAs were used: *tdc* (Acc. No. X67662), *str* (Acc. No. X61932), *hmgr* (Acc. No. M96068), *dxs* (Acc. No. AJ011840), *dxr* (Acc. No. AF250235), *mecs* (Acc. No. AF250236) and *g10 h* (Acc. No. AJ251269).

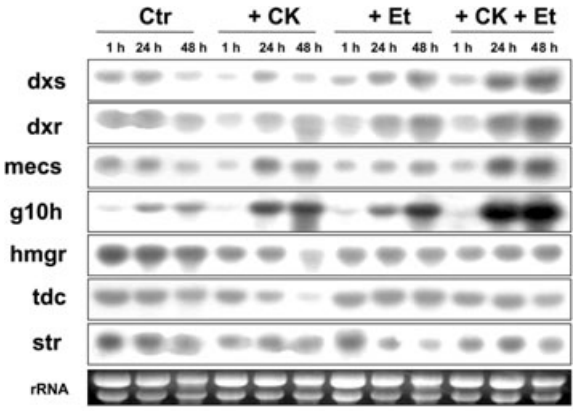

Fig. **4** Regulation of TIA biosynthetic gene expression by CK and ethylene in C20D cells grown in 2,4-D-free medium. The cells were treated on the 4th day of culture with 5 μM of *trans*-zeatin (CK), or 0.1 mM ethefon (Et) or both (CK + Et). They were harvested after 1, 24 or 48 hours of treatment. Ctr = untreated cells grown in 2,4-D-free medium (*dxs* and *dxr* expression is induced by 2,4-D suppression; *mecs*, *hmgr*, *tdc,* and *str* are already expressed in cells grown in the presence of 2,4-D [10]).

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